REDUCED EXTRACELLULAR ELECTRON SHUTTLES INCREASE HYDROGEN PRODUCTION AND SUBSTRATE UTILIZATION IN PURE CULTURE CLOSTRIDIUM BEIJERINCKII FERMENTATION

BY

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DISSERTATION

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ABSTRACT

Biological hydrogen production is considered one reasonable alternative for generating H_2 as a fuel that can be linked to electricity production in standard fuel cells. However, the molar yields are currently too low to be economically feasible and the inefficient production rates lead to the oversizing of bioreactors for possible industrial application. Lignocellulosic materials, the most abundant biomaterial on the earth, have been considered promising renewable substrates for biofuel production; however, better strategies need to be developed to increase sugar utilization in the fermentation process. This study aims to bridge the gap between the current status of biological hydrogen production and future industrial applications by developing an innovative physiological strategy (adding reduced electron shuttles) to increase the hydrogen production yield, kinetics and substrate utilization in a model hydrogen producing microorganism-*Clostridium beijerinckii* fermentation system.

Results show that adding extracellular hydroquinones (anthrahydroquinone disulfonate; AH_2QDS) increased the hydrogen yield by 24-37% and the extent of xylose utilization by up to 56% when xylose was the sole substrate. Electron mass balance suggested that the increase of hydrogen yield was correlated to the pathway shift from butyrate production pathway to the acetate production pathway. Electron flows at the pathway level were examined to show that reduced electron shuttles increased the utilization of reduced ferredoxin to generate hydrogen, which contributed to increased hydrogen molar yield. Adding reduced electron shuttles also stimulated the kinetics of hydrogen production from different substrates including xylose, glucose and cellobiose.

The increases of hydrogen production kinetics were consistent with the increases of substrate utilization rates and apparent growth rates. Further tests using mixed sugar at different glucose:xylose ratios demonstrated that adding reduced electron shuttles increased the total substrate utilization and hydrogen production by improving the xylose utilization, while concomitantly increasing the kinetics of hydrogen production and substrate utilization. Increasing concentrations of AH_2QDS increased hydrogen production and xylose utilization at fixed glucose:xylose ratio of 1:1.

The results from this study show that adding reduced electron shuttles stimulated the hydrogen production rate at all tested sugar systems, increased hydrogen molar yield in xylose fermentation, and increased the xylose utilization in mixed sugar fermentation. Possible mechanisms have been proposed to address the influence of reduced electron shuttles on the fermentative physiology. Results suggest that it might be promising to use reduced electron shuttles to develop better strategies to extract biohydrogen from the lignocellulosic materials by allowing us to manipulate the fermentative metabolism at the physiological level.

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CHAPTER 1 INTRODUCTION AND RESEARCH OBJECTIVES

Increasing demands on energy and fuels pose significant influence on the global economy, national security, and the sustainability of the whole human society. The aggressive utilization of fossil fuel has promoted the prosperity of industrialization in the past century; however, the decreasing stock amount of global fossil fuels and the deteriorating global warming caused by the carbon-based fuel combustion keeps ringing the warning bell to alert the human society of the unsustainable development (Antizar-Ladislao and Turrion-Gomez, 2008). With the increasing global population and energy demand, the current fossil fuel is projected to be exhausted within 60-100 years (Agardy and Nemerow, 2005).

Biofuels have aroused the interest of both scientific researchers and policy makers for decades. The first generation of biofuel, corn ethanol, has attracted worldwide debate. Even though plenty of criticism on the low energy balance and the global food crisis triggered by converting corn to fuel discredited the future of food-based fuel production as an alternative to fossil fuel (Ragauskas et al., 2006), the concept of biomass-to-fuel has successfully found its voice in the renewable energy market, which also led to the development of the second generation biofuel. The second generation biofuel makes use of lignocellulosic materials instead of food resources. Lignocellulosic materials are the most abundant biomaterials in nature and in agricultural and industrial waste (Taylor, 2008). Moreover, the development of other energy carriers beyond ethanol (e.g. hydrogen and butanol) further expands the applicability and sustainability of the biofuel.

Hydrogen, the cleanest energy resource, carries the foremost advantage over other carbon-based fuels: the combustion of hydrogen emits no greenhouse gas, which becomes especially important nowadays, as the human society is exerting the utmost endeavors to address the globe warming by limiting carbon dioxide emissions. The possible replacement of carbon-based fuel with hydrogen avoids emitting carbon dioxide, which might contribute to ultimately solving the issues related to global warming (Dorf, 2001).

Compared to the current energy-intensive, industrially-available physical/chemical approaches, biological hydrogen production is of more importance in terms of high energy yield and sustainability. Biological hydrogen production requires minimal energy input and maximizes the overall energy yield of hydrogen as an energy resource (Nath and Das, 2004). Moreover, hydrogen can be produced from a wide spectrum of renewable substrates including lignocellulosic materials and carbohydrates-rich wastes (Levin et al., 2009).

Hydrogen can be produced biologically by two major routes: dark fermentation and photosynthesis (Nath and Das, 2004). Dark fermentation possesses several advantages over photosynthesis in terms of potential industrial applications. Hydrogen has to be produced efficiently in order to be utilized in fuel cell technology, which is considered the ideal platform for hydrogen utilization as an energy carrier (Levin et al., 2004). Research to date has suggested that fermentative hydrogen production rates were not efficient, which could result in the oversizing of bioreactors to produce adequate hydrogen in fuel cells; not to mention the photosynthetic hydrogen production rates were several times lower than fermentative hydrogen production (Levin et al., 2004). Thus, the

influence of electron shuttles on the fermentative hydrogen production rates was explored in this research.

Another objective of this research is to improve substrate utilization of carbohydrates in the fermentative process. How to efficiently utilize lignocellulosic materials is one of the identified barriers to the development of second generation biofuel. Pretreatment breaks down the complex chemical structure of lignocellulosic biomolecule and hydrolysis yields a wide spectrum of carbohydrates (Levin et al., 2009; Margeot et al., 2009). Most industrial microorganism can efficiently ferment the hexose sugars (i.e. glucose) of the hydrolysates of the lignocellulosic materials; however, pentose (i.e. xylose) utilization remains a challenge in the fermentation process (Kongjan et al., 2009; Kumar et al., 2009; Ren et al., 2009). This research investigated the influence of reduced electron shuttling compounds on the substrate utilization of different carbohydrates in the pure culture *Clostridium beijerinckii* fermentation system.

Last but not least, this research also examined the influence of reduced electron shuttles on the hydrogen production yields. Low hydrogen yield has been considered the primary obstacle in the industrial application of biohydrogen (Bartacek et al., 2007; Nath and Das, 2004). Several approaches have been proposed to increase hydrogen yields including the rapid removal of headspace hydrogen, enhanced temperature, and applying external electricity (Logan et al., 2008; Wang and Wan, 2008); however, a majority of these approaches require further energy input. This research showed a novel physiological approach to increase hydrogen production yield from xylose by adding reduced electron shuttles. Results also demonstrated that adding reduced electron shuttles increased the hydrogen production rates and the extent of substrate utilization when

different carbohydrates including glucose, xylose, cellobiose and mixed sugars were utilized as the substrates in pure culture *Clostridium beijerinckii* fermentation.

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CHAPTER 2 LITERATURE REVIEW

In this chapter, the history and development of biofuels are first summarized to demonstrate the blooming development of biofuel research and industry in the past few decades. Characteristics of hydrogen as a clean energy carrier are then reviewed to support the standpoint that biohydrogen might be a perfect alternative to replace the traditional fossil fuels in the future. Current biological hydrogen production processes are further generalized to illustrate the advantages and disadvantages of fermentative hydrogen production. Practical constraints including hydrogen production rate, hydrogen molar yield and substrate utilization that significantly restrict the application of hydrogen production are summarized, and the current state of researches that have aimed to address these problems is also reviewed. Extracellular electron shuttling compounds are overviewed and the state of the art research on electron shuttling biotechnologies is also summarized.

2.1 OVERVIEW ON THE DEVELOPMENT OF BIOFUEL

Biofuel refers to any sort of energy carrier, extracted from biomass (organic matters) (DOE, 2008; NREL, 2009). Biomass is a reproducible resource that is widely distributed on the surface of the earth. Thus, biofuel is broadly accepted as a sustainable alternative to definite fossil fuels for the future (DOE, 2008, 2009).

As early as in the 19th century, people started converting the agricultural products such as peanuts and corn to vegetable oils (Luque et al., 2008). The vegetable oils originated from bioproducts were the archetype of the modern biofuel. The initiation of modern biofuel for automobiles was launched in the 20th century by Rudolph Diesel and

Henry Ford. Diesel demonstrated the first compression ignition engine at the World's Exhibition in Paris in 1898 using peanut oil (the antitype of biodiesel); and Ford designed the first automobile running on ethanol in the early 20th century (Luque et al., 2008).

The first surge of biofuel development appeared during World War II. Germany experienced great fuel shortages and promoted the development of bioalcohol. On that occasion, bioalchohols fermented from potatoes were blended with gasoline to fuel the military vehicles (Luque et al., 2008; Songstad et al., 2009). However, despite its peak development during World War II, biofuel was soon outcompeted by gasoline and other fossil fuels due to the rapid development of the petroleum industry. Petroleum based fossil fuels replaced the biofuels in the global market as a result of low price.

The oil crisis in 1970's revived the vitality of biofuels politically and economically (Songstad et al., 2009). Several governments realized the danger that solely relying on exported fuel posed on national security and economic development and promoted the secondary development of biofuels. This period of rapid development gradually ceased as the crude oil price in the global market declined to the normal values.

As the human society enters 21th century, sustainable problems, increasing demand for energy, and the eminent depletion of petroleum fossil fuels altogether have pushed forward the blooming development of biofuels. A significant amount of endeavors have been devoted to promoting the applicability of biofuels globally and domestically. United Nations International Biofuels Forum was launched to strengthen the global collaboration on biofuels on a worldwide scale. In the United States, the Bush and Obama Administrations have been dedicating tremendous endeavors in researching and commercializing the biofuels. In 2006, President Bush initiated the Advanced Energy

Initiative, which significantly increased the research funding for biofuels. Later Energy Independent Security Act and Conservation and Energy Act were passed by Congress and signed into law in 2007 and 2008, respectively. The legislation of energy laws and billions of funding from federal agencies have significantly boosted the prosperity of biofuel research and market. President Obama inherited and further advanced the federal investment on biofuels since he took office in 2008. In the American Recovery and Reinvestment Act of 2009, over 780 million dollars of the stimulus bill was dedicated to fundamental and applied research on biofuels (Dorf, 2001).

Table 2.1 Development of biofuels

Generation	Energy carrier	Feed stock	Technology
1st	Bioethanol, biodiesel, biogas	Sugar crops, and oil crops	Transterification, sugar fermentation
2nd	Biobutanol, biohydrogen, bioalcohols, biodiesel, bio DMF(2,5-Dimethylfuran), bio DME(Dimethyl ether)	Non-edible biomass, lignocellulosic biomass (i.e woods, straw, energy crops), agricultural and food waste	Pretreatment&fermentation, transterification& hydrogenation, gasification
3rd	Biobutanol, biohydrogen, bioalcohols, biodiesel, aviation fuels	Marine macro-algae, micro-algae	Transterification/pyrolysis, fermentation, photosyntheis

Table 2.1 summarizes the technical development of biofuels. The first generation biofuels (bioethanol, biodiesel) were produced from agricultural food crops, which have aroused explosive debates (Naik et al., 2010). Major criticism included competition on food crops leading to increases of the global food prices and unsustainability related to deforestation, water and land use (Sims et al., 2010; Williams, 2008). The second generation of biofuels was extracted from non-food feedstock, which significantly improved the sustainability of the first generation biofuels. Non-edible crops can be cultivated in alternative lands to avoid the interference with land dedicated to food crops (Luque et al., 2008). Therefore, the second generation biofuels have been gradually replacing the first generation of biofuel as the hot topic for both academic researchers and industrial investors. Third generation of biofuels-algae type biofuels-have also emerged recently to expand the primary substrate to algae (Ragauskas et al., 2006).

2.2 BIOHYDROGEN, THE FUTURE OF HYDROGEN AS A PROMISING ENERGY CARRIER

Among different bioenergy carriers in Table 2.1, hydrogen appears to be the most attractive energy carrier. As the sole non-carbon based bioenergy carrier, biohydrogen embodies the maximum value of sustainability and minimum environmental impacts. Hydrogen has been widely accepted as the ultimate solution to the global warming since the end use generates zero greenhouse gases (Williams, 2002). Hydrogen can also be converted to electricity in fuel cells with the highest conversion efficiency compared to other carbon-based energy carriers (Sorensen, 2005). Hydrogen also has the highest energy content per unit mass of the all fuels; its higher heating value is 141.9 MJ/kg, almost three times of gasoline. Moreover, the storage and transportation of hydrogen can be flexible in gaseous, liquid, or metal hydrides form. Hydrogen gas or liquid hydrogen is convenient for large-amount storage and air and space transportation, while the metal hydrides can be used at small scale for vehicle transportation (Agardy and Nemerow, 2005).

Currently hydrogen is mainly being produced from fossil fuels via physical/chemical routes. Nearly 90% of industrial hydrogen is produced by the reaction of natural gas or light oil steam fractions at high temperature. Coal gasification and electrolysis of water are the alternative industrial means for hydrogen production (Yang, 2007). All these processes are highly energy intensive. Current major industrial usage of hydrogen includes: (1) hydrotreating and hydrocracking in refineries to upgrade crude oil (hydrotreating and hydrocracking), (2) reagents in the chemical industry to synthesize various chemical compounds (such as ammonia and methanol), and (3) reduction or

protection gas in metallurgical processes (Agardy and Nemerow, 2005). The major reason that restricts hydrogen serving as energy carrier is that the high energy demands in the industrial hydrogen production processes significantly decreases the overall energy balance of hydrogen as an energy carrier (da Rosa, 2009). Biologically hydrogen production requires minimum energy input, thereby assuming the future for hydrogen as an alternative to fossil fuels.

Biohydrogen can be classified into two major categories: light-driven process and dark fermentation (Bartacek et al., 2007a; Das and Veziroglu, 2008; Hallenbeck and Benemann, 2002). The following review will provide detailed information on fundamentals and comparison of different processes.

2.2.1 Light-driven hydrogen production

Light-driven hydrogen production in general refers to biological hydrogen production with the benefit of solar energy, which can be further divided into three sub-categories: direct photolysis, indirect photolysis and photo-fermentation (Hallenbeck and Benemann, 2002).

Direct Photolysis

Hydrogen is produced via the split the water in direct photolysis: $H_2O \rightarrow H_2+1/2O_2$. The overall process is composed by both light and dark reactions. In the light reaction, solar energy is captured by chlorophyll (Chll) molecules to split the water into hydrogen and oxygen and generate ATP, which was further consumed to fix CO_2 into carbohydrates in the dark reaction (Yang, 2007). In the hydrogen producing pathway, reduced ferredoxin serves as the direct electron donor for hydrogenase to make

hydrogen molecule (Figure 2.1) (Yang, 2007). The direct photolysis system has been demonstrated in both cell-free chloroplast-ferredoxin-hydrogenase systems and green algae (i.e. *Chlamydomonas reinhardtii*) to produce hydrogen(Greenbaum, 1988; Hallenbeck and Benemann, 2002).

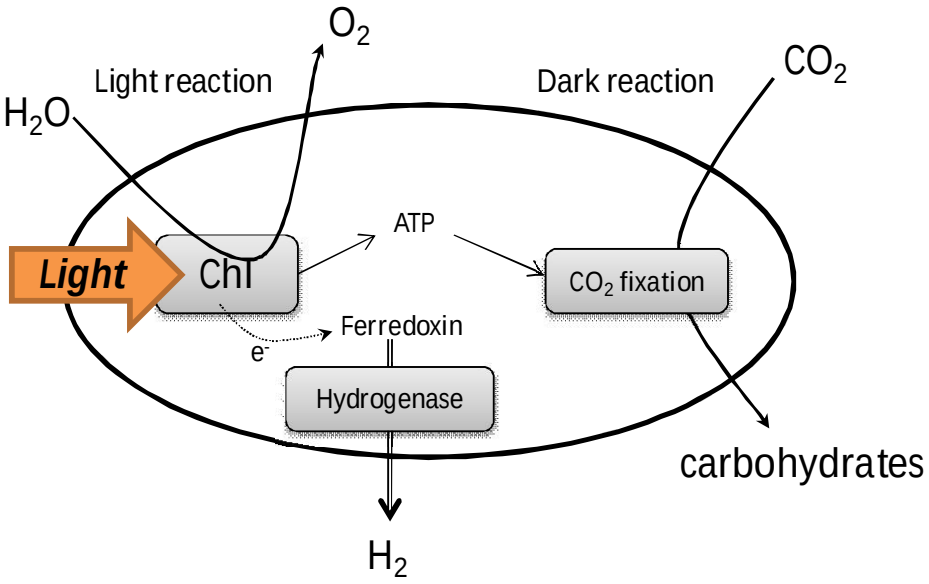

Figure 2.1 Direct biophotolysis in a green algae chloroplast

(adapted from (Yang, 2007))

Direct photolysis appears to be promising since the primary substrate for hydrogen is water, which is abundant on the earth. However, successful direct biophotolysis operation requires the oxygen partial pressure of approximate 1 atm, which significantly inhibits the activity of hydrogenase for hydrogen production.

Indirect Photolysis

Indirect photolysis is composed of two stages: oxygen evolution stage and hydrogen evolution stage. The separation of oxygen evolution from hydrogen production

successfully circumvents the oxygen sensitivity of the hydrogenase and supporting reducing cofactor system (Hallenbeck and Benemann, 2002). In the first stage, water is split into oxygen taking the benefit of light energy. Carbohydrates are further broken down in stage two to make hydrogen via hydrogenase pathway (Figure 2.2). Cyanobacteria are a group of photoautotrophic microorganisms that can produce hydrogen through the indirect photolysis (Angermayr et al., 2009).

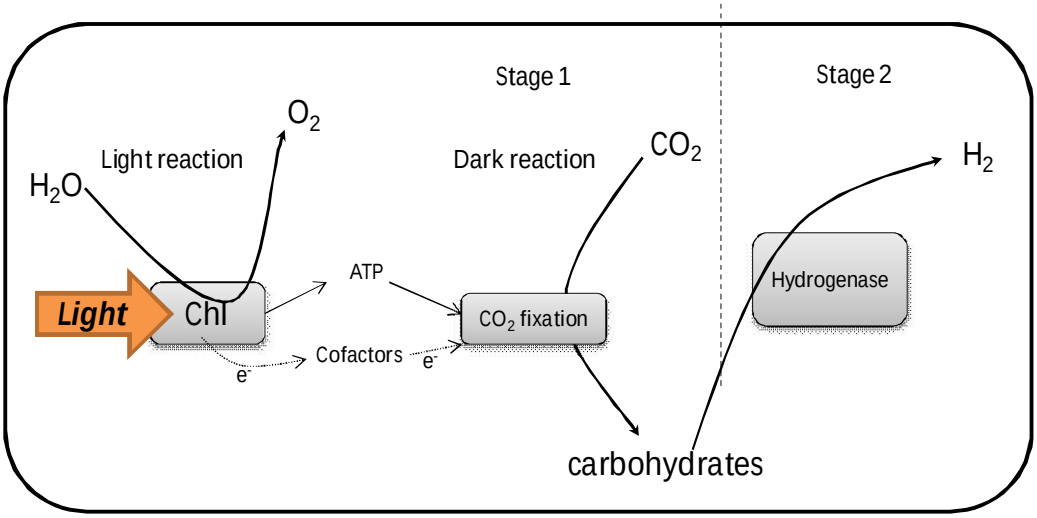

Fig. 2.2 Indirect biophotolysis for hydrogen production

Photo-Fermentation

In photofermentation, hydrogen is produced through actions of the nitrogenase system other than directly splitting of water (Hallenbeck and Benemann, 2002). Purple bacteria are one of the representative microorganisms for photofermentative hydrogen production. Purple bacteria can utilize organic acids or hydrogen sulfide as electron donors to generate ATP and reduce ferredoxin (Yang, 2007). Reduced ferredoxin is then oxidized by the nitrogenase system to produce hydrogen at the compensation of ATP (Fig. 2.3).

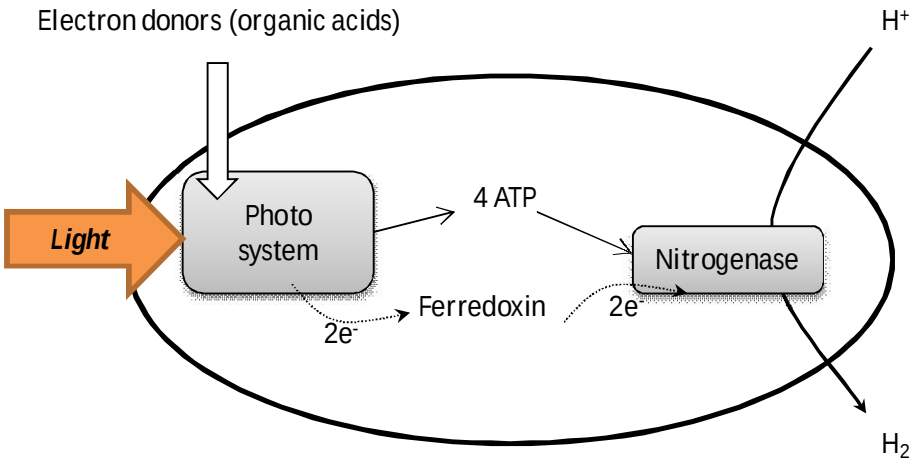

Fig. 2.3 Photofermentation for hydrogen production

2.2.2 Dark fermentation

Hydrogen can also be produced without light by anaerobic microorganisms grown on carbohydrate-rich substrates. Hydrogenase is the only enzyme that makes hydrogen. Even though the reaction is simple: $H^+ + e^- \rightarrow 0.5H_2$, hydrogenase is surprisingly complex. There are two basic types of hydrogenases: Fe-hydrogenases and Ni-Fe hydrogenases (Vignais and Colbeau, 2004). Available reducing equivalents have to be present other than hydrogenase to initiate the electron transfer to hydrogenase for hydrogen production. In dark fermentation, reducing equivalents are generated in substrate-level phosphorylation. The breakdown of carbohydrates to pyruvate is associated with the reduction of intracellular electron carriers. For example, NADH is generated in the glycolysis pathway; NADPH is generated in the Pentose-phosphate pathway. The further conversion of pyruvate is catalyzed by either one of the following enzyme systems for hydrogen production in so-far understood hydrogen producing microorganisms (Figure 2.4):

1. Pyruvate ferredoxin oxidoreductase: Pyruvate+CoA+Fd(ox) à Acetyl-CoA+Fd(red)

   Hydrogenase: Fd(red)+H^+ à H_2+Fd(ox)

2. Pyruvate formate lyase: Pyruvate+CoA à Acetyl-CoA+formate

   Hydrogenase: formate à H_2+CO_2

The strict anaerobes (i.e. *Clostridia*) derive hydrogen from Fd(red), while the enteric bacteria (such as *Enterobacter*) evolve hydrogen from formate (Hallenbeck, 2005; Levin et al., 2004).

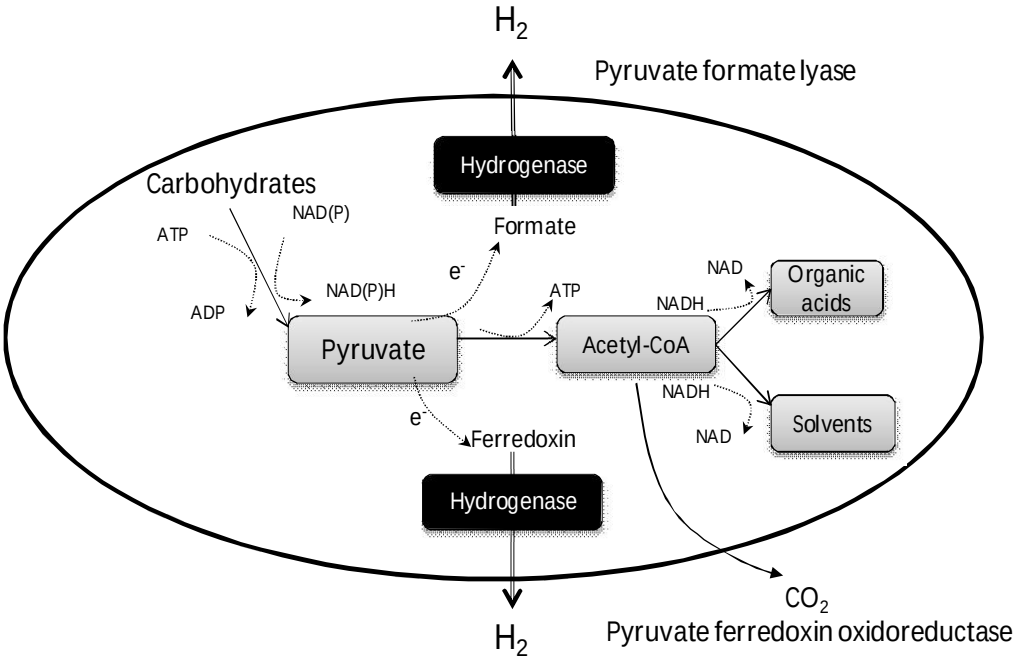

Figure 2.4 Fermentative hydrogen production

2.2.3 Comparison between photosynthetic and fermentative hydrogen production

Table 2.2 summarizes the differences between photosynthetic and fermentative hydrogen production processes. Direct and indirect photolysis use water as primary substrate; however, the oxygen sensitivity and low photochemical efficiency made them

hard to be applied industrially (Bartacek et al., 2007a; Yang, 2007). Photofermentative hydrogen production use organic acids as substrates, which can improve the overall yield of the whole process compared to dark fermentation. Yet, the efficiency of photo-fermentation process is extremely low, and the utilization of light greatly complicates the operation of the process (Levin et al., 2004). Thus, fermentative hydrogen production appears to assume the future of the biohydrogen, although there are still lots of problems that have to be addressed in both fundamental and applied researches (Bartacek et al., 2007a; Hallenbeck, 2009; Hallenbeck and Benemann, 2002; Hallenbeck and Ghosh, 2009).

Table 2.2 Comparison of different biohydrogen production processes

Process	Substrate	Pros	Cons
Direct photolysis	Water	Substrate	Oxygen toxicity
Indirect photolysis	Water	Substrate	Low photochemical efficiency
Photofermentation	Organic acid	Substrate, high yield	Low light utilization efficiency, low production rate
Dark fermentation	Carbohydrates	High efficiency, all-day hyrogen production regarless of light availabilty, easy to operate	Low yield; production rate not efficient enough for application; Substrate utilization

2.3 PRACTICAL PERSPECTIVE OF FERMENTATIVE HYDROGEN

Three key issues have been identified in various literature resources as significant barriers for fermentative hydrogen to be applied in large scale, including hydrogen molar yield, hydrogen production rate, and substrate utilization.

2.3.1 Hydrogen molar yield

Nearly all the review papers published in the recent 10 years on fermentative hydrogen production unanimously identified fermentative hydrogen production yield as a key issue that determines the industrial applicability of biohydrogen as a commercial fuel (Bartacek et al., 2007b; Chong et al., 2009; Das and Veziroglu, 2008; Hallenbeck, 2005; Hallenbeck, 2009; Hallenbeck and Benemann, 2002; Hallenbeck and Ghosh, 2009; Levin et al., 2009; Levin et al., 2004; Meher Kotay and Das, 2008; Nath and Das, 2004; Wang and Wan, 2009; Yang, 2007). In this review, fundamental microbiological constraints on hydrogen yield will be presented, followed by a summary of current researches dedicated to improving fermentative hydrogen yield.

Innate Metabolic Constraints On Hydrogen Yield

Theoretically, 1 mole carbohydrates (glucose) can be fermented to 12 mole hydrogen and 6 mole carbon dioxide:

$C_6H_{12}O_6+6H_2O \rightarrow 6CO_2+12H_2$ (Equation 2.1)

The maximum hydrogen yield can achieve 12 mole/mole glucose stoichiometrically; however, this reaction never takes place in realistic fermentation process even if it is thermodynamic favorable. To better understand the factors that control the hydrogen yields, metabolic carbon and electron flow have to be scrutinized at pathway level (Hallenbeck, 2005). Figure 2.5 shows the metabolic pathways for the representative hydrogen producing genus *Clostridium*.

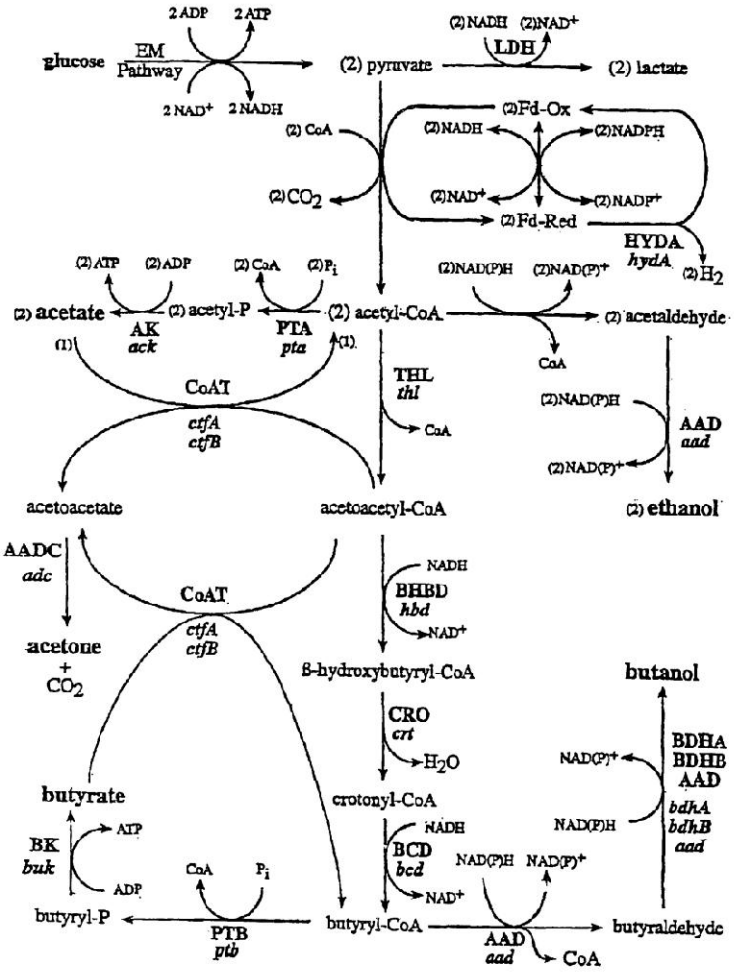

Fig. 2.5 Biochemical pathways of *Clostridium* fermentation

(www.freepatentsonline.com/6960465.html)

The direct constraint for hydrogen production is the available electron donors. Production of hydrogen is a reduction reaction; the redox potential for H^+/H_2 at neutral pH is -0.41 mV, which determines that only a few intracellular electron donors can be directly utilized by hydrogenase to produce hydrogen (Alberty, 2003). Reduced ferredoxin is a good electron donor with the lowest redox potential (-0.41 mV) among common intracellular carriers (Alberty, 2003). The reaction is not a strictly

thermodynamically favorable reaction at standard conditions, but it does take place when the hydrogen partial pressure is low. In this sense, the availability of Fd(red) is the factor that directly determines the hydrogen yield. Intracellular electron transport is very complicated, and the exchange of electrons among different electron carriers is common and catalyzed by different enzymatic system. For example, NADH:ferredoxin oxidoreductase catalyzes the exchange of electrons between NADH/NAD and Fd(red)/(ox) pools (White, 2000). There are also other enzymes that facilitate the electron switch between other electron carriers (e.g. NADP(H), FAD(H_2)) and ferredoxin. Therefore, available intracellular reducing equivalents (NADH and Fd(red)) provide electrons for hydrogen production, thereby determining the hydrogen production, since NADH can donate electrons to ferredoxin and then to proton to make hydrogen (Figure 2.5).

In dark fermentation, reducing equivalents (NADH) are first generated in the substrate level phosphorylation; and these reducing equivalents have to be regenerated to continue the cellular metabolism (Madigan and Martinko, 2006). Organic acid and solvent pathways are the primary choice of the cell to regenerate the cofactors, since the reaction of NADH with acetyl-CoA to make organic acids and alcohols is much more favorable than with proton to generate hydrogen. Therefore, fermentative hydrogen yield is constrained by organic byproduct production as competitors for reducing equivalents (Lee et al., 2008a).

Various Approaches To Increase Hydrogen Production Yield

Researchers have dedicated significant amount of efforts to increasing hydrogen production yields in the past 30 years. All the approaches can be classified into three

major categories: process optimization, metabolic engineering, and integrated technology.

(1) Process optimization:

i. Inoculum

Both pure culture based and mixed culture based hydrogen production have been widely investigated in the literature. *Clostridium* and *Enterobacter* are frequently reported hydrogen producing microorganisms in literatures (Chin et al., 2003b; Kim et al., 2008; Lin et al., 2007; Pan et al., 2008; Skonieczny and Yargeau, 2009; Wang et al., 2009; Zhang et al., 2009). *Clostridium* sp. is gram-positive, rod-shaped, strict anaerobes and spore-forming, and produce hydrogen via pyruvate-ferredoxin oxidoreductase-hydrogenase pathway; whereas *Enterobacter* are gram-negative, rod-shaped and facultative anaerobes (Mitchell, 1998; Wang and Wan, 2009). Mixed cultures from anaerobic sludge, activated sludge, compost and soil have been pretreated as inoculums for hydrogen production (Duangmanee et al., 2007; Mu et al., 2006; Rodriguez et al., 2006). The dominant microbial species after long operation time have been converged to Clostridium sp. in several mixed culture research (Duangmanee et al., 2007; Fang et al., 2002). Table 2.3 summarizes the hydrogen yields of various studies using both pure and mixed cultures. Although it appears that pure culture systems in general have high yield, sometimes hydrogen yield from mixed cultures can achieve equally high yields. It is also important to notice that in Table 2.3 the hydrogen yield values have varied considerably in the past studies. No conclusive decision has been achieved on whether mixed culture or pure culture systems assume higher yield.

Table 2.3 Hydrogen yield from different inoculum

Inoculum	Substrate	Operation	Yield	Ref.
Clostridium acetobutylicum	Glucose	Batch	2.0 mole/mole glucose	(Chin et al., 2003a)
Clostridium butyricum	Glucose	Continuous	1.4-2.3 mole/mole glucose	(Kataoka et al., 1997)
Clostridium beijerinckii	Glucose	Batch	2.86 mole/mole glucose	(Lin et al., 2007)
Clostridium butyricum	Glucose	Batch	2.29 mole/mole glucose	(Lin et al., 2007)
Clostridium pasteurianum CH4	Sucrose	Batch	2.07 mole/mole hexose	(Lo et al., 2008)
Enterobacter aerogens	Glucose	Batch	1.0 mole/mole glucose	(Yokoi et al., 1995)
Enterobacter cloacae IIT-BT 08	Glucose	Immobilized	2.3 mole/mole glucose	(Kumar and Das, 2001)
Mixed culture	Glucose	Continuous	1.43 mole/mole glucose	(Mizuno et al., 2000)
Mixed culture	Glucose	Continuous	1.58 mole/mole glucose	(Zhao et al., 2009)
Mixed culture	Xylose	Batch	1.3 mole/mole xylose	(Lin et al., 2008c)

ii. Temperature

It has been reported that increased temperature resulted in the increased hydrogen production yield in mixed culture fermentation. Wang and Wan demonstrated that increasing temperature resulted in the increased hydrogen production in an appropriate range (20-40 °C) (Wang and Wan, 2008). Munro et al. reported hydrogen production yield increased from 2.04 to 3.85 mole/mole substrate of pure culture *Thermotoga neapolitana* when the incubation temperature increased from 60 ℃ to 85 °C (Munro et al., 2009). Pakarine et al. also showed the highest hydrogen yield was achieved at 70 ℃ of all the tested cultivation temperature (35, 55 and 75°C) (Pakarinen et al., 2008). The

increased hydrogen yield at higher temperature is possibly due to the positive effect of temperature on the activation energy and thermodynamics (Hallenbeck, 2005). It has been pointed out that even though the optimal temperature reported significantly varied in literature, it fell into the mesophilic (37 ℃) and thermophilic (55 ℃) range (Wang and Wan, 2009).

iii. Substrate

Several studies have investigated the influence of substrate concentration/loading on the hydrogen production yield in batch/continuous system. However, there is no general conclusion whether high or low substrate concentration would result in the maximum hydrogen yield. Some research discoveries are in the favor of low substrate concentration: O-Thong et al. reported that the maximum hydrogen yield was achieved at the lowest substrate concentration tested (5.6 g COD/l) (O-Thong et al., 2008). Yu et al. demonstrated the same trend using the mixed culture with rice winery wastewater as the primary substrates (Yu et al.). There are also some findings that have shown substrate concentration at medium range resulted in the highest yield. Xing et al. reported a maximum hydrogen yield of 1.93 mole/mole glucose when substrate concentration is 10.7 g COD/l (tested range: 5.3-21.3 g COD/l) in a pure culture *Enterobacter* system (Xing et al., 2008). Van Ginkel et al. demonstrated that 7.5 g COD/l substrates yielded the highest hydrogen yield (tested range: 1.5-44.8 g/l) in mixed culture system (Ginkel et al., 2001). Some research also suggested that increased hydrogen yield was associated with enhanced substrate concentration (Lee et al., 2008b; Lo et al., 2008). There seems to be no general optimal substrate concentration in dark fermentation based on the literatures; the optimal substrate concentration is system specific.

iv. pH

pH has been identified as a key parameter that affects the hydrogen production yield, due to its influence on the enzymatic activity and bioenergetics (Hallenbeck, 2005; Lee et al., 2008a). It has been demonstrated that within an appropriate range increasing pH could lead to increased hydrogen yield, but further increase of pH, on the contrary, decreased the hydrogen yield (Wang and Wan, 2009). Like substrate concentration, pH is also system specific and needs to be optimized case by case to achieve maximum hydrogen yield. A wide range from 4.5 to 9.0 of pH have been reported in literature to have the maximum hydrogen yield in both pure culture and mixed culture system operated in both batch and continuous mode (Wang and Wan, 2009).

(2) Metabolic engineering

Research of metabolic engineering approaches to increase hydrogen yield has been so far focused on genetically traceable microorganisms. However, the progress has been limited due to poor knowledge of genetic information on existing pathways and intermediates, as well as the limited information on the substrate specificities of endogenous hydrogen production in relation to central metabolism (Jones, 2008). Despite of all these limitations, there are several published studies that have successfully increased fermentative hydrogen yields. Kumar et al. reported a substantial hydrogen yield increase (62%, 3.4 mole/mole versus 2.1 mole/mole) was achieved by selectively blocking organic acids and alcohol pathways in *Enterobacter cloacae* IIT-BT 08 (Kumar et al., 2001). Morimoto et al. also demonstrated that overexpression of a native hydrogenase in *Clostridium paraputrificum* successfully nearly doubled the hydrogen yield (Morimoto et al., 2005). Moreover, some progress has also been achieved on

predicting changes in metabolic flux that may increase the hydrogen yield, taking advantage of computational modeling (Jones, 2008). With more information of computational modeling approaches that generate optimal flux solutions, better metabolic approaches can be expected by deleting/overexpressing certain genes to enhance hydrogen production yield (Becker et al., 2007; Jones, 2008).

(3) Integrated technology

Several integrated processes have been developed to combine dark fermentation to improve the hydrogen yield. Two of the most important technologies include microbial electrolysis cell and combining dark fermentation with photosynthesis. Organic acid and solvents, which are inevitable byproducts in dark fermentation, can be fully converted to H_2 and CO_2 taking advantage of an external electrical input in the microbial electrolysis cell (Rittmann, 2008). This technology was demonstrated to increase hydrogen yield to approximately maximum theoretical value. Researches done by the Logan research group and Rittmann group have demonstrated the successful operation of microbial electrolysis cells of hydrogen production from acetate (Call and Logan, 2008; Call et al., 2009; Ditzig et al., 2007; Lee et al., 2009). Another technology that combines dark fermentation with photosynthesis has also been under development to improve the hydrogen yield, since some photosynthetic microorganisms (i.e. purple non-sulfur bacteria) can utilize organic acids as the substrate to produce hydrogen with the benefit of the solar light (Jones, 2008). *Rhodobacter sphaeroides* has shown its ability to use acetate to produce hydrogen with light in literature (Kars et al., 2009; Nath and Das, 2009). Other phototrophs such as *Chlamydomonas reihardtii* and *Rhodopseudomonas palustris* have also been demonstrated to produce hydrogen from such organic acids as acetate (Carlozzi and

Lambardi, 2009; Vijayaraghavan et al., 2009). Integration of the above photosynthetic microorganism with dark fermentation has been shown to increase the overall hydrogen yield in both single and dual processes (Nath and Das, 2009; Vijayaraghavan et al., 2009).

2.3.2 Hydrogen production rate

Levin et al. calculated the minimum hydrogen production rate to be applied in a fuel cell, which seems to be the most promising commercial application of hydrogen. The calculation indicated a hydrogen flow rate of 23.9 mole/h to fuel a 1 KW proton exchange membrane fuel cell (PEMFC) (Levin et al., 2004). However, the hydrogen production rate of dark fermentation, which has been shown to be around 100 times of the light-driven process, is reported in the range of 21.0-121.0 mole/L-h in literature. A brief calculation further revealed that to fuel a 1.0 KW fuel cell, the size of the bioreactor has to achieve $1.98*10^2$ to $1.14*10^3$ liter (Levin et al., 2004). The oversizing of the bioreactor, caused by the low efficiency of bioprocesses made the industrial application of hydrogen in the fuel cell impossible, even though hydrogen has the highest energy efficiency in fuel cells (Nath and Das, 2004). Thus, it is also important to increase hydrogen production rates to decrease the size of the bioreactor for commercial-scale hydrogen production.

Several researches have reported increased hydrogen production by optimizing the operation parameters: temperature, pH and substrate concentration. Elevated temperature is supposed to increase the hydrogen production rate due to the general influence of temperature on the enzymatic activity and chemical reactions (Hallenbeck and Ghosh, 2009). However, in literature, thermophilic microorganisms do not have higher hydrogen production rates than mesophilic microorganisms; often they are even

lower. The maximum hydrogen production rate by *Caldicellulosiruptor saccharolyticus*, an extreme thermophile, on sucrose was 8.4 mmole/L-h, which was far less than that by mesophilic *Clostridium*, which was 21.03 mmole/L-h in literature (Levin et al., 2004; Niel et al., 2003; Taguchi et al., 1996). Lin et al. also identified the same negative influence of temperature on hydrogen production using a *Clostridium* dominated mixed culture system (Lin et al., 2008c). So far, published research data have suggested that mesophilic hydrogen producing microorganisms may bear higher hydrogen production rate than thermophilic microorganisms (Levin et al., 2004; Lin et al., 2008c).

Substrate concentration was suggested to influence the hydrogen production rate in various studies (Kargi and Pamukoglu, 2009; Lin et al., 2008a; Sharma and Li, 2009). Sharma and Li showed that increased substrate concentration resulted in the elevated hydrogen production rate; however, further increase of substrate concentration on the contrary had the reverse effect on the hydrogen production rate. They further illustrated the inhibitory effect of high substrate concentration on hydrogen production (Sharma and Li, 2009). Kargi and Pamukoglu reported the same trend of medium range substrate concentration resulted in maximum hydrogen production in that relatively lower and higher substrate concentration yielded low hydrogen production due to substrate limitation and product inhibition, respectively (Kargi and Pamukoglu, 2009). Therefore, research to date supports that the medium substrate concentration likely results in the maximum hydrogen production rate (Kargi and Pamukoglu, 2009; Lin et al., 2008a; Sharma and Li, 2009).

Last, but not least, pH significantly affects hydrogen production rate, possibly due to its influence on the enzymatic activity (Zheng and Yu, 2004). It has been shown that

there is no general conclusion on which range of pH results in the most efficient hydrogen production rate in previous studies. Sharma and Li reported the optimal pH of 5.89 when maximum hydrogen production rate was achieved in mixed culture hydrogen production (Sharma and Li, 2009). Sivaramakrishna et al. and Lin et al. supported the acidic range of pH for maximum hydrogen production rate (Lin et al., 2008b; Sivaramakrishna et al., 2009). However, there is also published research data that favors the pH range of 6.5 to 7.0 for maximum hydrogen production rate. Jo et al demonstrated the pH of 6.5 resulted in the maximum hydrogen production rate of 5089 ml H_2/g/l in pure culture *Clostridium tyrobutyricum* JM1 fermentation (Jo et al., 2008). Zhang et al. also showed the initial pH of 7.0 brought about the maximum hydrogen production rate in the mixed culture system (Zhang et al., 2007).

2.3.3 Substrate utilization

Since the lignocellulosic conversion has been accepted as the future for biofuels; how to efficiently utilize lignocellulosic materials has aroused general interest among biofuel researchers. The pretreatment followed by fermentation has been considered the promising way to convert lignocellulosic raw materials to diversified energy carriers (Antizar-Ladislao and Turrion-Gomez, 2008; Saxena et al., 2009; Taylor, 2008). Various pretreatment methods such as acid/alkali hydrolysis and ammonia fiber explosion similarly yield a wide spectrum of hexose and pentose (Kumar et al., 2009). The mixed sugar is composed primarily of glucose and xylose, as well as arabinose and mannose (Demirbas, 2008). Six-carbon sugars such as glucose can be efficiently fermented by industrial microorganisms (e.g. *Saccharomyces cerevisiae*) to bioethanol, yet substrate utilization of pentose (xylose) is not as efficient as that of hexose, and most of the

industrial microorganisms are not capable of using pentose as the primary substrates (Kongjan et al., 2009; Kumar et al., 2009; Ren et al., 2009). As a result, it is vital to investigate and increase the ability of fermenting microorganisms to utilize the whole range of available carbohydrates from lignocellulosic biomass hydrolysis (Kumar et al., 2009).

In literature, various approaches have been employed to increase the substrate utilization for different microorganisms, especially for pentose. Mixed culture processes appear to carry more advantages in terms of utilizing mixed carbohydrates compared to pure culture based technology, due to the contribution of diversified microbial community for the mixed culture. However, even with mixed cultures, pentose utilization efficiency still remains challenging. Kongjan et al. reported only 44% of xylose was fermented to hydrogen when the initial concentration was 4 g/l in mixed extreme thermophilic culture (70 °C) (Kongjan et al., 2009). Prakashma et al. also suggested mixed anaerobic consortia utilized approximate 60% of xylose at high xylose concentration in the batch reactor (Prakasham et al., 2009).

Metabolic engineering approaches have been widely explored to strengthen xylose utilization. Lots of papers have reported increased carbohydrate utilization using recombinant gene technology such as overexpressing xylukinase and enhancing pentose-phosphate-pathway (Alper and Stephanopoulos, 2009; Matsushika et al., 2009). Overexpression of endogenous transaldolase gene has been demonstrated to improve growth on xylose in *S. cerevisiae* due to the improved pentose-phosphate pathway (Jin et al., 2005). Deleting the gene encoding NADPH-dependent aldehyde dehydrogenase resulted in improved xylose fermentation rate (Sonderegger et al., 2004). Expression of

the talA gene encoding transaldolase from *E. coli* also improved the xylose utilization in *Clostridium acetobutylicum* as a result of enhanced pentose phosphate pathway (Cu et al., 2009).

2.4 EXTRACELLULAR ELECTRON SHUTTLING

Electron shuttling compounds are essential components in intracellular electron transfer that facilitate various redox biochemical reactions in cellular metabolism. Intracellular electron shuttling compounds, also referred to as electron carriers, are organic molecules that can accept and donate electrons for multiple metabolic reactions. For example, nictotinamide adenine dinucleotid (NAD^+), ubiquinone, and cytochromes are indispensable electron carriers in the Q cycle of the electron transport chain (White, 2000). Like intracellular electron carriers, extracellular electron shuttling compounds (EES) bear a similar function and can be oxidized and reduced in multiple redox reactions without being decomposed. Extracellular electron shuttling compounds that have been investigated in literatures include methy viologen, neutral red, AQDS, riboflavin, phenazine, juglone, Lawsone, humic substances and more (Watanabe et al., 2009). The review paper by Van der Zee and Cervantes summarized the common characteristics of electron shuttling compounds: a midpoint redox potential for both reduced and oxidized reaction, solubility and ability to cross the cell membrane (Van der Zee and Cervantes, 2009). Watanabe et al. supported that electron shuttling compounds might be membrane-permeable and may affect cellular energy metabolism (Watanabe et al., 2009).

Research to date on extracellular electron shuttling mainly focus on bioremediation and biodegradation areas such as azo dye decolorization, dechlorination and removal of inorganic pollutants, as well as microbial fuel cells (Thrash and Coates, 2008; Van der Zee and Cervantes, 2009; Watanabe et al., 2009). Artificial electron shuttling compounds effectively lower the activation energy of chemical reactions, as a result stimulating the overall kinetics. Electron shuttling compounds have been demonstrated to significantly accelerate azo-dye reduction, thereby decreasing the size of the bioreactor for the azo-dye removal from the wastewater. The dye/EES ratio was suggested to be the important designing factor for the process. Research on electron shuttling mediated dechlorination, mostly focused on carbon tetrachloride as the primary pollutant, demonstrated the optimal concentration of EES (in the range of 10-100 μM) and the impact of the environmental parameters such as pH and temperature. Electron shuttling compounds have also been widely investigated in microbial fuel cells to improve the overall efficiency of power outputs and substrate utilization rates in the literature (Thrash and Coates, 2008).

The influence of electron shuttling compounds on the fermentation and biofuel production has been moderately studied in literature, mainly by the Soucaille and Zeikus research group. Girbal et al. reported that adding neutral red modified carbon and electron flow in *Clostridium acetobutylicum* fermentation by deviating electron flow from hydrogen to NADH production initiated by the enzymatic down-regulation of hydrogenase and up-regulation of ferredoxin NADH oxidoreducatse (Girbal et al., 1995). Peguin et al. demonstrated a similar function of methyl viologen addition in batch *Clostridium acetobutylicum* fermentation. Electrons were diverted from hydrogen

pathways to generate excessive reducing power, which further induced the overproduction of butanol (Peguin and Soucaille, 1995). They also showed the metabolic perturbation induced by the electron carrier addition resulted in a reduction of growth rate and an increase of the lag phase (Peguin et al., 1994). Emde and Schink studied the influence of electrochemically reduced electron shuttling on glucose fermentation by pure culture *Propionibacterium freudenreichii* and identified that the presence of reduced electron shuttling compounds increased the propionate formation from 73% to over 90% (Emde and Schink, 1990). Schuppert et al. also demonstrated a similar result by shifting propionate formation from 68% to 100% during lactate fermentation with *P. acidi-propionici* by adding electrochemically reduced electron shuttling compounds (Schuppert et al., 1992). Park and Zeikus introduced neutral red as an electron donor shuttle and successfully improved glucose utilization, cell yield, ethanol, and succinate production in pure culture *Actinobacillus succinogenes*. They also proposed that neutral red may replace the membrane-bound menaquinone in the electron transport chain (Park et al., 1999; Park and Zeikus, 1999).

Even though electron shuttling compounds have been suggested to influence fermentation end products and kinetics, few researches have been done to study its influence on hydrogen production. Only two papers that have been published investigated the influence of AH_2QDS addition and NAD/NADH addition on hydrogen production (Chong et al., 2009; Hatch and Finneran, 2008). The influence of reduced electron shuttling compounds on hydrogen production have been investigated by Hatch and Finneran to show increased hydrogen production by adding reduced electron shuttling compounds in the cell suspension system (Hatch and Finneran, 2008). This is the first to

report the influence of reduced electron shuttling compounds on fermentative hydrogen production. Zhang and co-workers investigated the influence of extracellular NADH/NAD^+ addition on hydrogen production by *Enterobacter aerogenes*, and proposed that the external NADH potentially affected the electron flow across the membrane and eventually influenced the hydrogen evolution (Chong et al., 2009). Since two different hydrogen production pathways (formate lyase-hydrogenase and ferredoxin oxidoreductase-hydrogenase) coexisted in the *Enterobacter aerogenes* cellular metabolism and the cells can generate/regenerate NADH/NAD intracellularly, the influence of extracellular NADH/NAD was not specifically addressed in the paper.

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CHAPTER 3 REDUCED EXTRACELLULAR ELECTRON SHUTTLES INCREASE HYDROGEN MOLAR YIELD AND XYLOSE UTILIZATION IN GROWING CULTURES OF CLOSTRIDIUM BEIJERINCKII

3.1 ABSTRACT

H_2 production and xylose utilization were investigated using the fermentative culture *Clostridium beijerinckii* NCIMB 8052. Adding the reduced form of the quinone/hydroquinone electron shuttle anthrahydroquinone-2,6-disulfonate (AH_2QDS) increased the extent of xylose utilization by 56% and hydrogen molar yield by 24-37%. Enhanced hydrogen molar yield correlated with increased xylose utilization and increases in the acetate/butyrate product ratio. The acetate/butyrate (A/B) ratio increased while the intracellular NADH/NAD^+ ratio decreased as a result of AH_2QDS addition. An electron balance indicated that AH_2QDS shifted the electrons from the butyric acid pathway (NADH dependent pathway) to the acetic acid pathway (non-NADH dependent pathway), putatively creating a "surplus" of reducing equivalents that were then available for hydrogen production. These data demonstrate that hydrogen yield and xylose utilization can be manipulated by amending redox active molecules into growing cultures. This will impact biohydrogen/biofuel production by allowing physiological manipulations of growing cells for increased (or decreased) output of selected metabolites using amendments that are not consumed during the reactions. Although the current yield increases are small they suggest a target for cellular alterations. In addition, the increased xylose utilization will be critical to fermentation of pre-treated lignocellulosic feedstocks, which may have higher xylose content.

Key words: hydrogen production, hydrogen molar yield, xylose utilization, electron shuttling compounds

3.2 INTRODUCTION

A number of substrates have been investigated for fermentative hydrogen production including defined carbohydrates (mainly glucose and sucrose) (Kotsopoulos et al., 2006; Lee et al., 2008; Lee and Rittmann, 2009; Lin et al., 2007; Skonieczny and Yargeau, 2009), undefined waste materials such as lignocellulosic biomass (Kapdan and Kargi, 2006; Lalaurette et al., 2009; Levin et al., 2009) and agricultural wastes (Duerr et al., 2007; Kapdan and Kargi, 2006; Vijayaraghavan et al., 2007). Lignocellulosic biomass cannot be efficiently fermented by most industrial microorganisms, and needs to be pretreated (Kumar et al., 2009). Glucose and xylose are the two most abundant monomers resulting from lignocellulose pretreatment (Kumar et al., 2009; Ren et al., 2009). Xylose is also the second most abundant carbohydrate in agricultural waste (Temudo et al., 2009). Hexose molecules such as glucose are effectively fermented by various industrial microorganisms (i.e *Saccharomyces cerevisiae*, *Clostridium acetobutylicum*) to generate bio-energy carriers (e.g. bioethanol) at high yields (Alper and Stephanopoulos, 2009; Kongjan et al., 2009). However, xylose utilization is generally less efficient for most, if not all, industrial microorganisms in pure or mixed culture fermentation (Kongjan et al., 2009; Temudo et al., 2009).

Clostridium beijerinckii NCIMB 8052 is a fermentative, hydrogen-producing microorganism that uses several substrates (Lin et al., 2007; Skonieczny and Yargeau, 2009). Although *C. beijerinckii* can ferment xylose to produce hydrogen, xylose is utilized less efficiently than glucose (Mitchell, 1996).

Hydrogen molar yield is widely accepted as a key issue in determining the industrial applicability of bio-hydrogen as an alternative energy carrier (Nath and Das, 2004).

Hydrogen production in *Clostridium* fermentation is a reaction that takes electrons from intracellular electron carriers (reduced ferredoxin, Fd(red)) and reduces protons to H_2 to expend reducing equivalents and regenerate oxidized carriers such as NAD^+. In dark fermentation, reducing equivalents are first generated during substrate level phosphorylation. These reducing equivalents have to be regenerated to form different end products including organic acids, solvents and hydrogen to maintain cellular metabolism and energy balances (Madigan and Brock, 2009). In this regard fermentation product formation is merely a redistribution of reducing equivalents and hydrogen molar yield is influenced by the electron equivalents from the organic acid/solvent pathway versus those that are available for ferredoxin and hydrogenase (Lee et al., 2008). It is this "redistribution" that we aim to influence by amending extracellular hydroquinones.

Electron shuttles are a group of redox active molecules that cycle between oxidized and reduced states in both chemical and biological systems (Hatch and Finneran, 2008; Hernandez and Newman, 2001; Lovley et al., 1996; Lovley et al., 1999; Roden et al., 2010; Schwarzenbach et al., 1990). Extracellular electron shuttling has been primarily investigated with respect to Fe(III) reduction in bioremediation (DiChristina et al., 2002; Hernandez and Newman, 2001; Lovley, 1991; Lovley et al., 1998); with few studies addressing its influence on fermentation (Bhushan et al., 2006; Borch et al., 2005; Girbal et al., 1995). Studies with *C. acetobutylicum* demonstrated the influence of the electron shuttling compounds Neutral Red and Methyl Viologen on the overall metabolism of *Clostridium* fermentation and proposed that adding the oxidized form extracellular electron shuttling compounds shifted the electron and carbon flow to favor solvent production by modulating the intracellular $NADH/NAD^+$ ratio and regulating the activity

of several key enzymes such as NADH-ferredoxin oxidoreductase and glyceraldehyde dehydrogenase (Girbal et al., 1995; Peguin and Soucaille, 1995). Hatch and Finneran demonstrated that reduced electron shuttling compounds improved hydrogen yield in resting cell suspensions of *C. beijerinckii* (Hatch and Finneran, 2008). Zhang et al. (2009) reported that extracellular NADH and NAD^+ stimulated hydrogen production rate and yield for *Enterobacter aerogenes*.

In this study we quantify the influence of the reduced electron shuttle (hydroanthraquinone-1,6-disulfonate, AH_2QDS) on *C. beijerinckii* NCIMB 8052 fermentation with xylose as the sole substrate. Electron balances were estimated for the fermentation intermediates to provide putative mechanisms for the overall effect of reduced extracellular shuttles on xylose utilization and hydrogen molar yield.

3.3 MATERIALS AND METHODS

Culture Maintenance

Clostridium beijerinckii NCIMB 8052 (ATCC number 51743) was obtained from TetraVitae Bioscience, Inc, Champaign, IL. The culture was revived from frozen spores in TYG medium (Tryptone 3 g/L, yeast extract 2 g/L and glucose 1 g/L), and was transferred to Modified P2 medium each week (Baer et al., 1987). The culture was incubated at 37 ℃ (Hatch and Finneran, 2008). The growth conditions in Modified P2 medium were 2 g/L $(NH_4)_2SO_4$, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $MnSO_4$, 0.01 g/L NaCl, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 1 mg/L p-aminobenzoic acid, 1 mg/L thiamine, and 0.01 mg/L biotin (Baer et al., 1987).

Batch experiments

Cells were grown in anoxic (anoxic operationally defined as zero oxygen concentration; this is differentiated from anaerobic which refers to the actual cellular metabolism) pressure tubes with fixed aqueous and headspace volumes (total aqueous volume of 10 mL). Modified P2 medium (Baer et al., 1987) was used with varying AH_2QDS and substrate concentrations to identify the influence of AH_2QDS on hydrogen production and xylose utilization and fermentation. Headspace and aqueous samples were collected using anoxic, sterile syringes and needles. Tubes were incubated at 37°C, without shaking, in the dark. All batch testes were run in triplicate.

Chemicals

Anthraquinone-2,6-disulfonate (AQDS) was purchased from Sigma-Aldrich (Milwaukee, WI). To generate AH_2QDS, a bicarbonate (final concentration 30mM) buffered 5 mM AQDS solution was bubbled with 80:20 H_2:CO_2 for at least 1 hour in the presence of 100 g/L palladium-coated Aluminum catalyst (Sigma) and then incubated at 30 ℃ overnight . Reduced anthraquinone-2,6-disulfonate (AH_2QDS) stock solution was then bubbled with hydrogen-free, nitrogen gas and double filtered with 0.2 micron filter (PALL Acrodisc® syringe filter) into a sterile, evacuated pressure tube. Hydrogen was not present in the stock solution.

Analytical Techniques

0.5 mL gas headspace samples were collected and injected into a GC (Shimadzu GC-14A) with a carbosieve S-II Spherical Carbon Column (Supelco, Bellefonte, PA) equipped with a Thermal Conductivity Detector (SRI instrument Model 110). Nitrogen was the carrier gas; the temperatures of the detector and oven were set as 150 and 50 °C,

respectively. Cell growth was monitored by measuring optical density (OD) at 600 nm (Hatch and Finneran, 2008).

Aqueous samples were collected with sterilized syringes and filtered through 0.2 micron sterile PTFE syringe filters. The samples were analyzed for organic acids (acetate, butyrate) at 210 nm using an HPLC (Dionex Summit) system equipped with a Transgenomic organic acid column; and for solvents (acetone, butanol and ethanol) using GC-FID (Shimadzu-GC2014) with an Agilent DB-FFAP capillary column (Temperature was set at 40 ºC for 3 minutes and increased to 220 ºC at the rate of 50 ºC /min). Xylose concentration was quantified using the phenol-sulfuric method (Taylor, 1995).

Electron Distribution Analysis

An electron balance was quantified based on fermentation end products, which included acetate, butyrate, hydrogen and biomass. The electrons transferred to each product were calculated as E(products)=mass of the product*electron equivalents of product. Total electrons from the primary substrates (E(substrates)=mass of substrate consumption*electron equivalents of xylose) were used to normalize the electron transfer to obtain the specific electron distribution. Electron equivalents per mole acetate, butyrate, hydrogen, biomass and xylose were shown in the supporting information.

Electron flow analysis and ATP calculation were performed using the electron distribution and were based on the modified electron flow model, which was originally proposed by Lee et al. (Lee et al., 2009). Several operational definitions were added to those that have already been reported for glucose fermentation systems:

(i) 1 mole of xylose is fermented to 1.67 mol pyruvate and 1.67 mol NADH;

(ii) A total amount of 1.67 mole ATP was generated via substrate-level phosphorylation for every mole xylose fermented;

(iii) 1 mole butyrate and acetate production generates 1 mol ATP via Ack/Pta pathway and Bck/Pta pathway, respectively.

We subtracted the reducing equivalents of NADH from the electron equivalents of organic acid products (acetate and butyrate) to get the reducing equivalents as acetyl-CoA. Reducing equivalents of Fd_{red} were further calculated by subtracting the reducing equivalents of acetyl-CoA from pyruvate. Since in pure culture *C. beijerinckii* fermentation hydrogen is produced only via Fd:hydrogenase system, we estimated the amount of Fd_{red} that goes to hydrogen production from the hydrogen data. We further estimated the electron exchanges between NADH and reduced ferredoxin.

3.4 RESULTS

Cell Growth and Hydrogen Production

Both hydrogen production and cell density reached their maxima at approximately 40 hours after inoculation in all tested conditions (Figure 3.1). Hydroquinone amendment increased normalized hydrogen production and biomass concentration, while the quinone form (oxidized control) did not alter growth or normalized hydrogen production relative to unamended cells (Figure 3.1). Both biomass yield and normalized hydrogen production (cumulative moles produced per mole xylose added) increased in a manner directly proportional to increased initial concentrations of AH_2QDS (Figure 1a and 1b).

When AH_2QDS was not present, normalized cumulative hydrogen was approximate 0.85 mole/mole xylose (cumulative hydrogen of 114 μmole per test tube). Adding 100

μM AH_2QDS increased the normalized hydrogen production by 28% to 1.03 mole/mole xylose added (146 μmole per test tube). Increasing of AH_2QDS concentrations to 250 and 500 μM also resulted in 57% and 107% increase of hydrogen production and 16% and 55% increase of biomass concentration as compared to non-AH_2QDS amended controls, respectively.

Xylose Utilization and Hydrogen Yield

Approximately 50% of the xylose was consumed at the end of growth in tubes that were not amended with AH_2QDS (Figure 3.2). Adding 100 μM AH_2QDS did not significantly increase the extent of xylose utilization; however, increasing AH_2QDS concentration to 250 and 500 μM improved average xylose utilization by 12% and 56%, respectively, relative to unamended controls. Hydrogen molar yield also increased as a result of AH_2QDS addition; the yield was 1.60 mol hydrogen/mol xylose without AH_2QDS; the yields were 2.07, 2.25 and 2.09 mol hydrogen/mol xylose when amended with 100, 250 and 500 μM AH_2QDS, respectively.

Electron Distribution Into Fermentation End Products

The electron distribution was calculated based on end products (biomass, acetate, butyrate and hydrogen), and as well as the acetate/butyrate ratio at different AH_2QDS concentrations. The butyrate pathway consumed the largest proportion of electrons (70-79%) for all tested conditions; however, AH_2QDS shifted the electrons from butyrate production to acetate production (Figure 3.3). When AH_2QDS was not present, approximately 4% and 79% of electrons were distributed to acetate and butyrate pathway, respectively. Adding AH_2QDS increased the electron distribution to acetate from approximately 4% to 6-9%, while simultaneously decreasing the electron distribution to

butyrate from approximately 79% to 70-75%. The resulting acetate/butyrate ratio shifts ranged from 0.14 mole/mole to 0.30 mole/mole, with higher ratios at 100 and 250μM, and a slight decrease to 0.2 at 500 μM. In all cases the acetate:butyrate ratios for AH_2QDS-amended cells were higher than unamended or AQDS amended cells (Figure 3.3).

Electron Flow from Reduced Ferredoxin To Hydrogen Production

Table 3.1 summarizes the estimated electron equivalents of intermediate products (acetyl-CoA) and intracellular carriers (NAD^+ and reduced ferredoxin) amongst all treatments using a modified electron flow model (Collins et al., 1994; Lee et al., 2009). Reduced ferredoxin can be utilized bi-directionally to produce hydrogen by hydrogenase or reduce NAD^+ to NADH by NAD^+-ferredoxin reductase. In the electron flow model, 0.11 meq^- NADH was utilized to reduce ferredoxin and thenby hydrogenase to produce hydrogen in the absence of AH_2QDS; this value increased to 1.18-1.78 $meq^-$when AH_2QDS was amended at concentrations from 100 to 500 μM. ATP yields also increased from 2.66 to 2.79-2.83 moles per mole substrate by adding AH_2QDS from 100 μM to 500 μM.

Influence of substrate concentration on hydrogen production and xylose utilization

Xylose concentration was scaled from 0.2 to 5g/L in the presence and absence of a fixed 250 μM AH_2QDS concentration (Figure 3.4). When xylose concentration was below 1 g/L, normalized cumulative hydrogen production was similar at the end of fermentation process irrespective of AH_2QDS addition, reaching 2.5 and 2.6 mole/mole xylose added (32 and 85 μmole hydrogen per incubation) at initial xylose concentration

of 0.2 g/L and 0.5 g/L, respectively. There was a significant difference in the normalized total hydrogen production between incubations without AH_2QDS and tubes with AH_2QDS when xylose concentrations were 1 or 5 g/L. Normalized hydrogen production productions were 1.6 mole/mole xylose added (108 µmole of hydrogen per incubation) without AH_2QDS and 2.1 mole/mole xylose added (152 µmole of hydrogen per incubation) with 250 µM AH_2QDS at a xylose concentration of 1 g/L, and was 0.3 mole/mole xylose added (113 µmole of hydrogen per incubation) without AH_2QDS and 0.5 mole/mole xylose added (171 µmole of hydrogen per incubation) with 250 µM AH_2QDS at a xylose concentration of 5 g/L (Figure 3.4).

The hydrogen molar yield was not significantly different in either the 0.2 or 0.5g/L xylose incubations, irrespective of the presence or absence of AH_2QDS (Figure 5). Hydrogen molar yield increased between 18 and 24% when AH_2QDS was added at xylose concentrations ranging from 1 to 5g/L (Figure 3.5). When xylose concentration was low (below 1 g/L), over 92% of the xylose was consumed in the end of the fermentation process; however, only 35-50% of the xylose was utilized at xylose concentrations above 1 g/L.

3.5 DISCUSSION

These results demonstrated that adding the reduced form of an electron shuttling molecule (a hydroquinone in this case) increased hydrogen molar yield by 25% and xylose utilization by 56%. The acetate:butyrate ratio increased as a response to AH_2QDS amendment, which affected intracellular ATP yields and the electron flow from NADH to reduced ferredoxin for H_2 synthesis (Table 3.1). Increases in xylose utilization are

critical because this is a limiting factor for pretreated hemicellulose feedstocks that are higher in xylose content than glucose content. These data will allow us to develop strategies for manipulating substrate utilization and the generation of fermentation end products.

These data can be contrasted with our past results with glucose-grown *C. beijerinckii* (Hatch and Finneran, 2008). Previous work indicated a hydroquinone-concentration dependent increase in total hydrogen relative to cells that lacked hydroquinone, but the yield (mole H_2 per mole glucose) increase was only significant when 5 nM extracellular NAD^+ was added to medium. In the absence of exogenous NAD^+, the yields were the same irrespective of hydroquinone amendment. While growth rates with glucose were comparable to xylose, the final glucose-biomass yields were smaller in the presence of the hydroquinone. The primary difference, however, was the acetate:butyrate ratio; butyric acid increased slightly and acetic acid remained constant with glucose as the sole fermentable substrate. This further indicates that increasing the acetate:butyrate ratio contributes to increased hydrogen molar yield. In fact, in past experiments the added NAD^+ may have played as critical a role as the hydroquinone with respect to excess hydrogen production (Hatch and Finneran, 2008; Berríos-Rivera et al, 2002).

C. beijerinckii ferments xylose to 3-P-glyceraldehyde via the Pentose phosphate pathway (Figure 6). 3-P-glyceraldehyde is further oxidized to acetyl-CoA to generate ATP, coupled to the reduction of intermediate electron carriers (NAD^+ and NADP). H_2, organic acids (acetate and butyrate), and solvents (acetone, butanol and ethanol) are produced to balance the intermediate reducing equivalents (reduced ferredoxin and NADH) that are generated during substrate-level phosphorylation (White, 2000). Soluble

metabolites such as acetic and butyric acid are the major constraint on H_2 yield, since the pathways are competing for reducing equivalents (Lee et al., 2008; Rodriguez et al., 2006; White, 2000).

Adding AH_2QDS increased acetate and decreased butyrate production (Figure 3.3). Acetate and butyrate production pathways branch at the point after acetyl-CoA (Figure 3.6). Acetate is produced from acetyl-CoA by Ack/Pta pathway:

$$\text{Acetyl-CoA+Pi+ADP} \rightarrow \text{Acetate+ATP+CoA}$$

This reaction is catalyzed by phosphotransacetylase and acetate kinase, and does not require direct input of electrons from reduced electron carriers (White, 2000). Butyrate is produced from acetyl-CoA by several steps and the overall reaction is:

$$2\text{Acetyl-CoA+2NADH+Pi+ADP} \rightarrow \text{Butyrate+2NAD}^{+}\text{+ATP+CoA}$$

Producing 1 mole of butyrate consumes two moles of NADH (White, 2000). The data demonstrated that the decrease in butyrate and increase in acetate resulted in an increased amount of reduced intracellular NADH, which must be expended to maintain the balance between reduced and oxidized electron carriers. The most reasonable alternate pathway is via ferredoxin to generate molecular H_2.

Clostridium beijerinckii uses reduced ferredoxin as the electron donor for the hydrogenase system, and ultimately H_2 synthesis (Gheshlaghi et al., 2009). Reduced ferredoxin is produced either by pyruvate-ferredoxin oxidoreductase from decarboxylation of pyruvate or by NADH-ferredoxin oxidoreductases with NADH as the electron donor. NADH-ferredoxin oxidoreductase can also catalyze the reverse reaction to reduce NAD^+ with reduced ferredoxin as electron donor (Figure 3.6). Therefore,

reducing equivalents can flow to ferredoxin and from ferredoxin, depending on what cellular metabolism needs at the time. Normally the activity of NADH-ferredoxin reductase outcompetes the ferredoxin-NAD^+ reductase reductase, which means more reducing equivalents flow from NADH to reduce ferredoxin for hydrogen production (Crabbendam et al., 1985; van Andel et al., 1985). Adding AH_2QDS increased the electron flow from NADH to reduced ferredoxin, thereby increasing the hydrogen production (Table 3.1). This is based on the electron balance calculated for cells in the presence and absence of AH_2QDS. This also explains the approximately 25% hydrogen molar yield increases when AH_2QDS was added to the cells. More hydrogen is produced per mole of carbon consumed because electron flow was redirected from an electron-consuming pathway (butyrate) to an electron neutral pathway (acetate); this in turn left excess NADH in the system during a growth phase when NADH is rapidly oxidized to maintain cellular activity. The excessive reducing equivalents were possibly utilized to reduce ferredoxin (Table 3.1), ultimately leading to increased hydrogen. In addition to the mechanism proposes here, increased xylose utilization may also have contributed to increased hydrogen production, which is discussed further below.

AH_2QDS also increased the xylose utilization by up to 56%. Approximately 0.9-1.8 g/L of xylose was fermented when AH_2QDS was not present at the initial substrate concentration of 1-5 g/L. Neither the substrate concentration nor product concentration exceeded the threshold level of inhibition (Ezeji et al., 2004), and nutrient concentration did not inhibit the substrate utilization (data not shown), it was possible that the limited xylose utilization in unamended was due to either limitations with intracellular co-factors

or incomplete xylose uptake across the cellular membrane, as previously reported (Girbal et al., 1995; Guedon et al., 1999; Temudo et al., 2009).

Xylose is taken up via active transport, meaning that translocation across the cell membrane consumes ATP. Inefficient xylose uptake and utilization is a critical issue for pre-treated lignocellulosic feedstocks, and strategies to increase xylose transport and/or utilization are necessary to increase the efficiency of pre-treated material with high xylose:glucose content. Xylose transport in bacteria is not generally linked to the phophotransferase system (Mitchell, 1998), and ATP availability (for active transport) was reported to be the controlling factor in xylose uptake (Cook et al., 1994; Sonderegger et al., 2004; Temudo et al., 2009; Zhou et al., 2009). AH_2QDS increased the intracellular ATP yields at all levels tested; while the increases were modest even ATP in slight excess of typical values may be used for cellular processes, including xylose transport. While it is still speculative it deserves further investigation, because increased ATP availability due to hydroquinone amendment may contribute to increasing xylose utilization (Zhou et al., 2009).

Understanding the distribution of reducing equivalents and intracellular carbon is important in regulating the product formation for fermentation processes (Berríos-Rivera et al., 2002). Manipulating intracellular molecules by amending exogenous compounds has been investigated as a possible method to alter fermentative metabolism to generate higher yields of desired products, which is what we are attempting here (Heux et al., 2006; Sánchez et al., 2005). The hydroquinone AH_2QDS increased hydrogen yield, and xylose utilization, while increasing acetate:butyrate ratio during acidogenic growth of *Clostridium beijerinckii*. Although further investigation is needed to define the

mechanism(s) by which this molecule exerts its effects, we used electron flow models to postulate that alterations in the acetate:butyrate ratio increased the reducing equivalents available from reduced ferredoxin for H_2 synthesis. This was most likely due to shifts in intracellular NADH, but evaluating this hypothesis requires more data. In addition, there were small but significant increases in ATP, which is required to actively transport xylose across the cell membrane. The results of this study demonstrate the potential of using electron shuttles to redirect carbon and electron flow to targeted pathways in fermentative metabolism.

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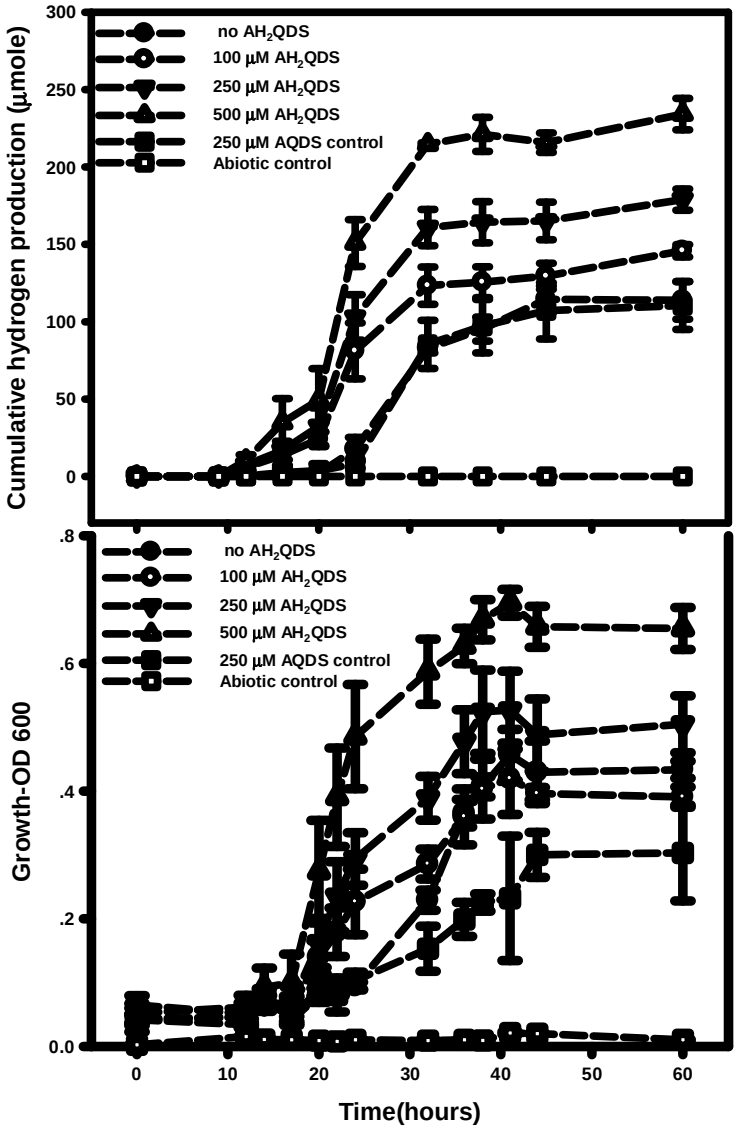

Figure 3.1 Cumulative hydrogen production (a) and cell growth (b) as functions of time at different AH_2QDS concentrations. Initial xylose concentrations were 2 g/L. Data points are the means of triplicate analyses. Bars indicate one standard deviation.

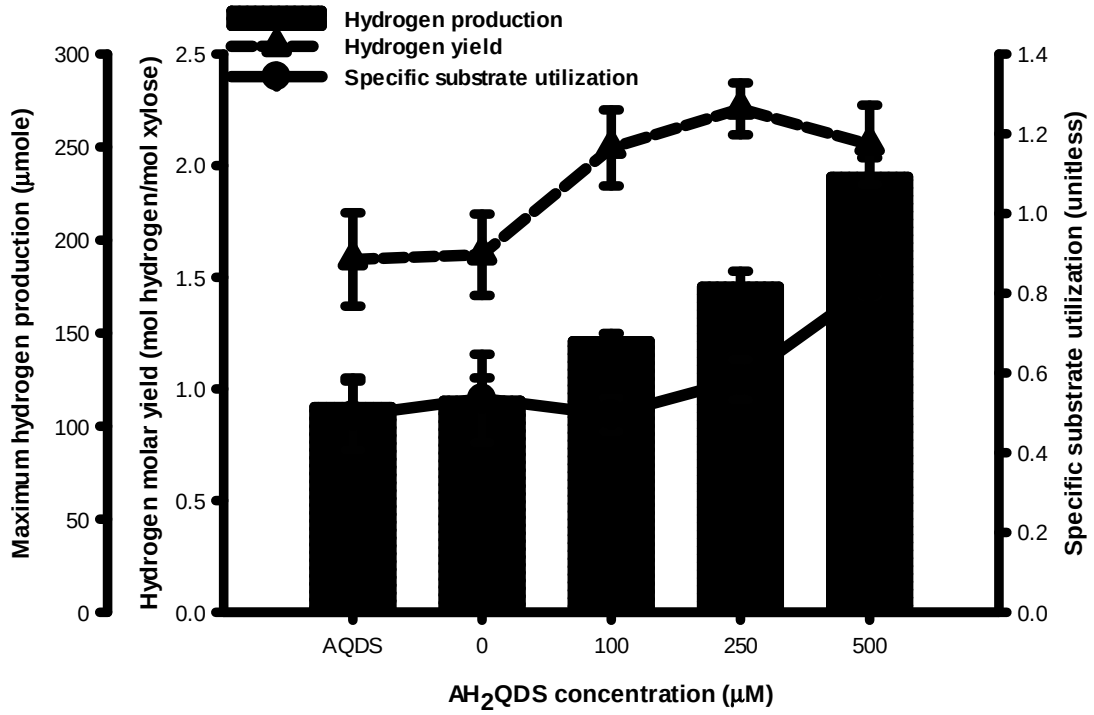

Figure 3.2 Hydrogen molar yields, specific substrate utilization and maximum hydrogen production at different AH_2QDS concentrations. Hydrogen molar yield was calculated as the maximum hydrogen production/substrate consumed. Specific substrate utilization was calculated as xylose consumed/initial xylose. Maximum hydrogen production represents the maximum of cumulative hydrogen production. Results are the means of triplicate experiment. Bars indicate one standard deviation.

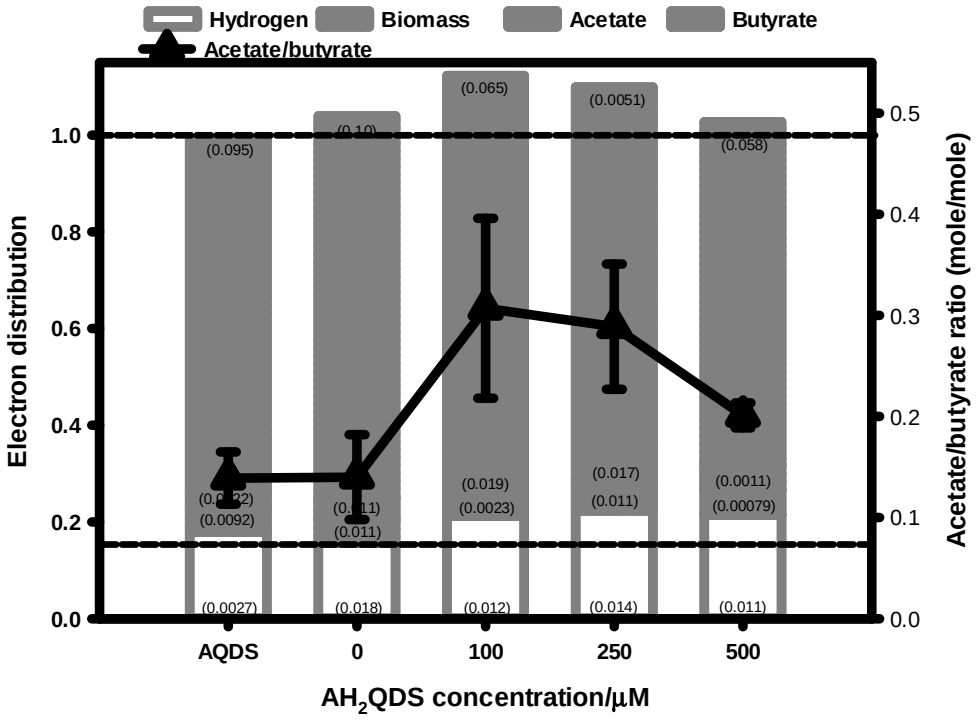

Figure 3.3 Electron distribution and acetate/butyrate ratio as functions of AH_2QDS concentration. Values in parentheses are the standard deviations for each product based on replicate values. Electron distribution was calculated as electron equivalents*the amount of the products normalized by total electrons donated by primary substrates. See supporting materials for more information.

Table 3.1 Electron flow analysis of fermentative intermediates

Intermediates and products	No AH_2QDS	100 µM AH_2QDS	250 µM AH_2QDS	500 µM AH_2QDS
Acetate+Butyrate (meq e^-)	16.88	17.32	16.76	16.72
NAD^+-ox[a] (meq e^-)	3.20	3.08	3.00	3.12
Acetyl-CoA (meq e^-)	13.68	14.24	13.76	13.60
Fd_{red} (meq e^-)	3.02	2.46	2.94	3.10
H_2 (meq e^-)	3.13	4.24	4.46	4.28
NADH-Fe_{red} [b](meq e^-)	**0.11**	**1.78**	**1.52**	**1.18**
ATP [c]	**2.66**	**2.83**	**2.82**	**2.79**

Units are e- meq

[a]Electrons consumed as NADH from Acetyl-CoA to butyrate

[b] Electrons from NADH to Fd_{red} and then to hydrogen production

[c]ATP estimation was based on the A/B ratio

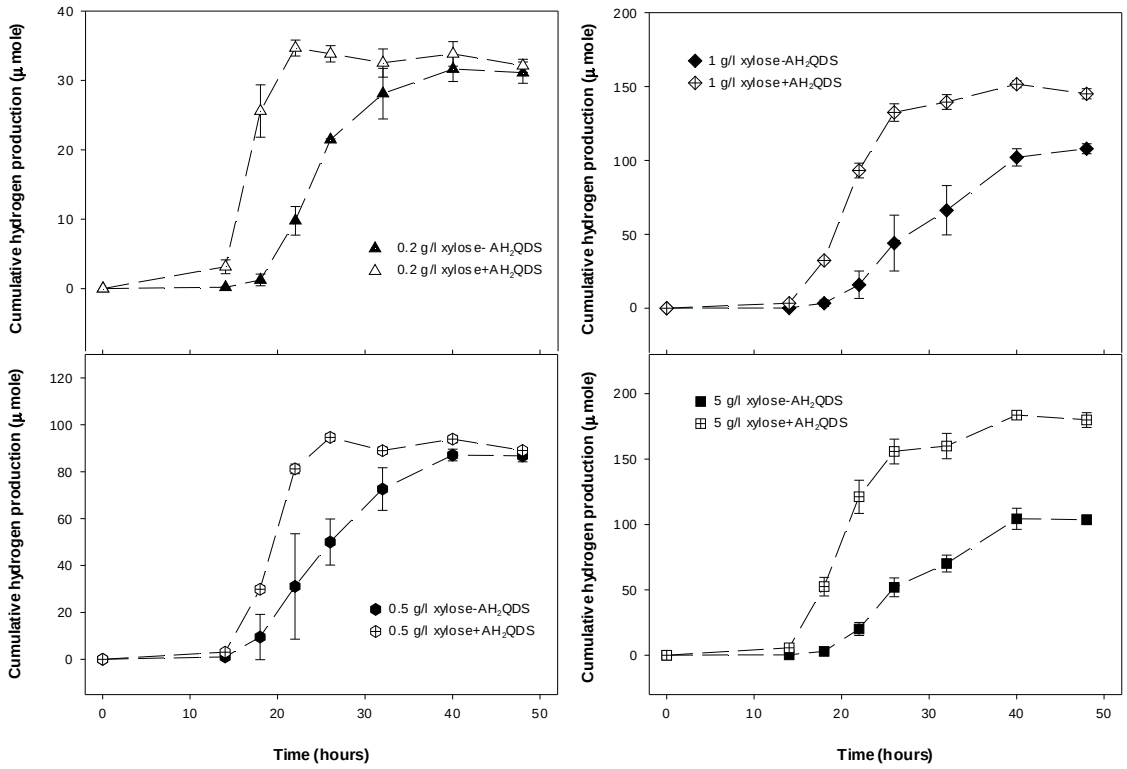

Figure 3.4 Cumulative hydrogen production as a function of time at different substrate concentrations plus/minus AH_2QDS addition. Data points are the means of triplicate analyses. Bars indicate one standard deviation.

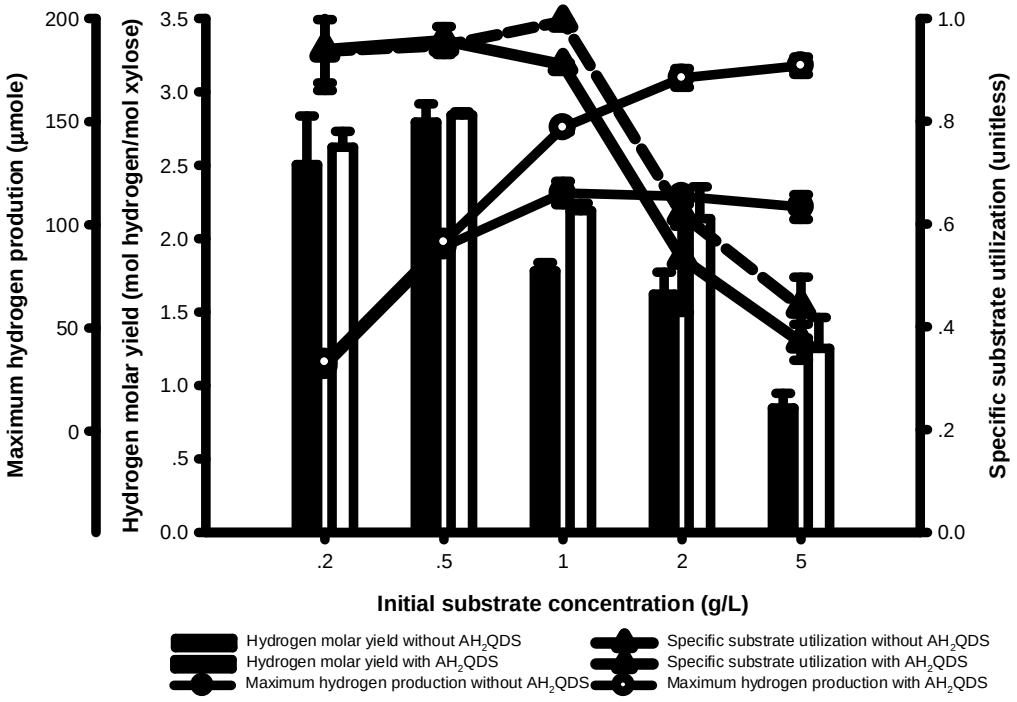

Figure 3.5 Maximum hydrogen production, hydrogen molar yields and specific substrate utilization at different initial substrate concentrations plus/minus AH_2QDS. Results are the means of triplicate experiment. Bars indicate one standard deviation.

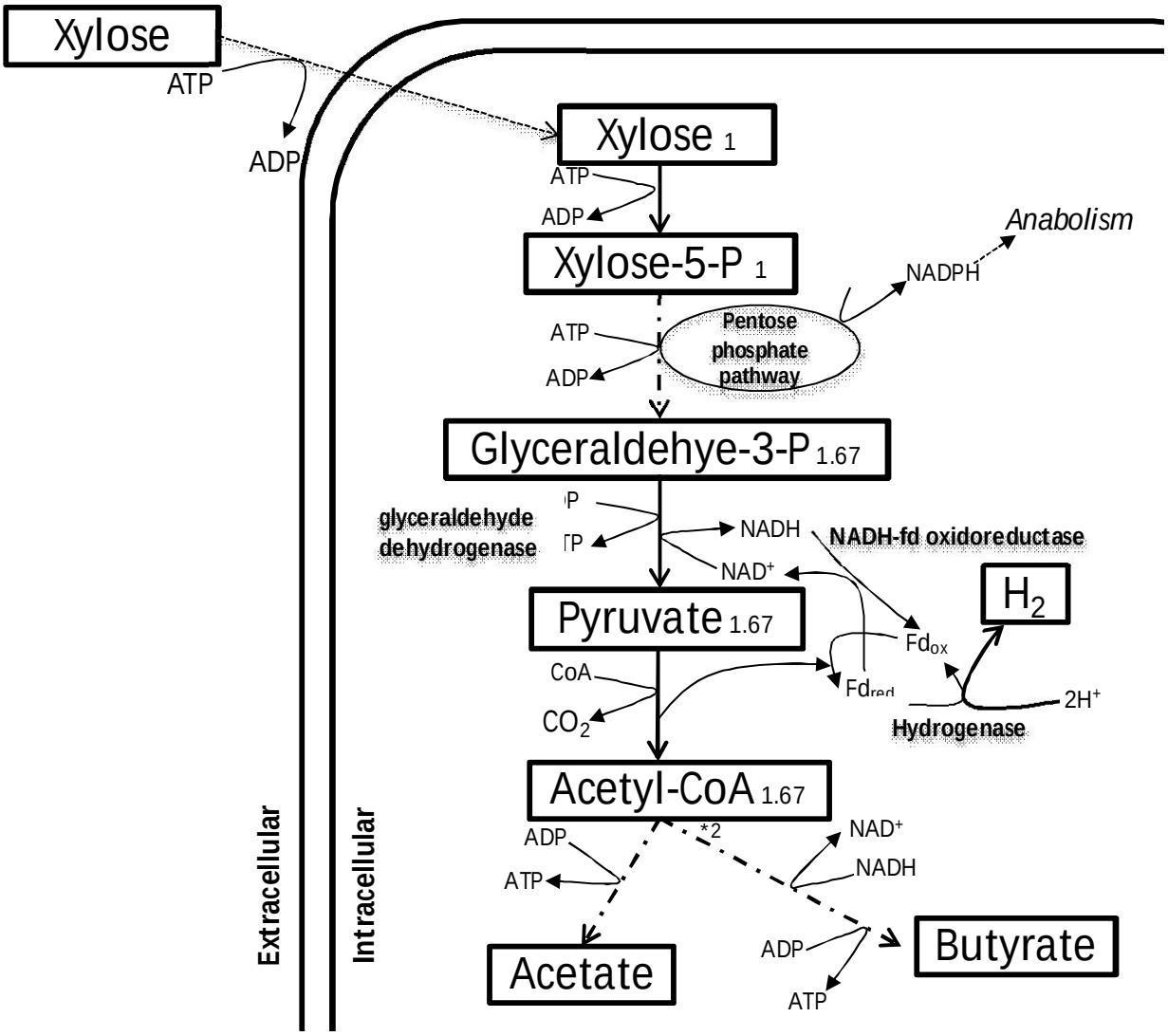

Figure 3.6 Adapted schematic of xylose fermentation in acidogenic *Clostridium beijerinckii* fermentation (Temudo et al., 2009). Numbers indicate the stoichiometric coefficient of intermediates from 1 mole xylose.

CHAPTER 4 REDUCED ELECTRON SHUTTLES INCREASE THE KINETICS OF HYDROGEN PRODUCTION IN PURE CULTURE CLOSTRIDIUM BEIJERINCKII FERMENTATION

4.1 ABSTRACT

Fermentative hydrogen production is considered a reasonable alternative for generating H_2 as a fuel that can be linked to electricity production in standard fuel cells. The kinetics of hydrogen production from glucose, xylose and cellobiose were investigated using pure culture *Clostridium beijerinckii*. Adding the reduced electron shuttles (anthrahydroquinone disulfonate; AH_2QDS) from 100 μM to 500 μM increased hydrogen production rate by 99%, 100% and 56% relative to unamended controls, when glucose, xylose, cellobiose were utilized as the primary substrates, respectively. Adding reduced electron shuttles (AH_2QDS) also resulted in the increase of substrate utilization rate and biomass growth rate by up to 1.24 and 0.9 times, respectively. These findings suggested that adding reduced electrons shuttles might influence the global cellular metabolism to increase the hydrogen production rate. Cell suspension experiments were conducted to investigate the influence of the reduced electron shuttles (AH_2QDS) on the hydrogen production rate from glyceraldehyde 3-phosphate, which is a shared intermediate in both glycolysis and pentose-phosphate pathway. Current data demonstrated the 36-47% increase of hydrogen production rate in resting cell suspension when glyceraldehyde 3-phosphate was the sole carbon source, which implied that adding reduced electron shuttles (AH_2QDS) might increase the conversion rate from glyceraldehyde 3-phosphate in the central metabolism, thereby increasing the hydrogen production rate in *Clostridium* fermentation. These data demonstrate that adding the reduced electron shuttles increased hydrogen production rate in pure culture

Clostridium fermentation, and allow us to develop efficient hydrogen production strategy by engineering the fermentative metabolism at physiological level.

Key words: hydrogen production rate, AH_2QDS, reduced electron shuttles, *Clostridium beijerinckii*, glucose, xylose, cellobiose

4.2 INTRODUCTION

Among all the biofuels that have been proposed, biohydrogen is of great importance as a non-carbon based energy carrier (Hallenbeck, 2009). Biohydrogen can be generated by two major routes: dark fermentation and photosynthesis (Nath and Das, 2004). Dark fermentation appears to be practically more promising in that photosynthetic hydrogen production requires efficient utilization of light, which poses challenges on the reactor configuration, and the rates are 20-200 times lower than fermentative hydrogen processes (Lee et al., 2008; Levin et al., 2004; Nath and Das, 2004). Several bacterial genus have been reported to produce hydrogen such as *Clostridium and Enterobacter*. *Clostridium* genus has been reported to have higher yield values than *Enterobacter* genus and to be dominant in various mixed culture hydrogen production studies (Duangmanee et al., 2007; Fang et al., 2002; Lin et al., 2007). Fang et al. reported the dominant microbial clones of the hydrogen-producing granular sludge were affiliated with *Clostridium* genus (Fang et al., 2002). Duangmanee et al. also suggested that the *Clostridium* genus dominated the microbial community in a mixed-culture hydrogen producing reactor, and the abundance is directly related to the reactor performance of hydrogen production (Duangmanee et al., 2007). Furthermore, Lin et al. studied the hydrogen production of different *Clostridium* species, and showed that *C. beijerinckii* had the highest hydrogen production rate and yield among four tested *Clostridium* strains (Lin et al., 2007). In this study, *Clostridium beijerinckii* NCIMB 8052 was used as a model hydrogen production microorganism to investigate the influence of reduced electron shuttles on hydrogen production kinetics.

The future of sustainable biofuel production has been agreed to be dependent upon the ability of utilizing complex substrates in biomass and wastes other than food-related carbohydrates (Ghosh and Hallenbeck, 2009). Lignocellulosic biomass is the most abundant raw

biomaterial in the world and has been accepted to be a promising and low-cost feedstock for hydrogen production (Ren et al., 2009a). Pretreatment and enzymatic hydrolysis are necessary prior to fermentation to saccharify the lignocellulosic material into sugars due to the low efficiency of the direct fermentative conversion of lignocellulosic feedstock (Margeot et al., 2009). Pretreatment removes lignin and breaks down the complex structure of lignocellulosic feedstock, thereby allowing hydrolytic enzymes access to cellulose and hemicellulose (Margeot et al., 2009). Enzymatic hydrolysis of the cellulosic portion of lignocellulosic biomass yields two major products: glucose and cellobiose, which can be further converted to energy carriers in fermentation (Yeh et al., 2010). Moreover, a considerable amount of xylose is also produced from the pretreatment and hydrolysis of the hemicellulose portion of lignocellulosic biomass (Kumar et al., 2009). Xylose is reported to assume around 30-45% of the total carbohydrates from the pretreatment of corn stover, wheat straw and sugarcane bagasse hemicelluloses (Ahring et al., 1996; Kumar et al., 2009; Lavarack et al., 2002). In this study, glucose, xylose and cellobiose were investigated as the primary substrates to study the influence of reduced electron shuttling compounds on hydrogen production.

Low hydrogen yield and production rate have been suggested to be two major obstacles that restrict the industrial level of application of hydrogen as an energy source (Levin et al., 2004; Nath and Das, 2004). Various endeavors have been attempted to increase hydrogen molar yield, with much less attention made to the kinetics of biological hydrogen production (Call and Logan, 2008; Liu et al., 2005). A feasibility study conducted by Levin et al. suggested that kinetics of fermentative hydrogen production was not efficient enough to be practically feasible in fuel cell technology, where hydrogen bears the highest energy efficiency (Levin et al., 2004). They further calculated that a bioreactor with a volume of approximate 5700 L was necessary to power

a 5.0 kW fuel cell using the pure culture of mesophilic *Clostridium* sp. (Davila-Vazquez et al., 2009; Levin et al., 2004). A few studies have been reported to improve hydrogen production rate in literature (Davila-Vazquez et al., 2009; Levin et al., 2004). Thermophilic fermentative microorganisms have been reported to have higher hydrogen production rate than mesophilic bacteria; however, increased temperature scarifies the overall energy balance of the process by increasing the energy demands to heat (Hallenbeck, 2009). Rapid removal of hydrogen was reported to exert a positive influence on the hydrogen synthesis rate; however, rapid removal decreased hydrogen content in the gas phase, thereby increasing the cost of downstream separation and increasing the energy input associated with rapid removal. In this study, reduced electron shuttles were introduced in the batch fermentation system to improve the hydrogen production rate.

Extracellular electron shuttles have been widely investigated in bioremediation to stimulate the electron donor/acceptor utilization, with little attention paid to fermentative metabolism (DiChristina et al., 2002; Hernandez and Newman, 2001; Lovley, 1991; Lovley et al., 1998). Electron shuttles are redox active and can donate and accept electrons without being consumed in microbial metabolism (Hatch and Finneran, 2008; Hernandez and Newman, 2001; Lovley et al., 1996; Lovley et al., 1999; Roden et al., 2010; Schwarzenbach et al., 1990). Several published findings have demonstrated the influence of oxidized electron shuttling compounds on the solvent production of *C. acetobutylicum* fermentation (Girbal et al., 1995; Peguin and Soucaille, 1995). However, few if any data are available that show how reduced electron shuttles affected hydrogen production kinetics in fermentative metabolism. Our previous work demonstrated that adding reduced electron shuttling compounds (anthrahydroquinone disulfonate, AH_2QDS) increased hydrogen production in resting cell suspension systems (Hatch and Finneran, 2008).

This research is a continuance of the previous study and systematically investigated the influence of adding reduced electron shuttles (anthrahydroquinone disulfonate, AH_2QDS) on hydrogen production kinetics with different substrates including glucose, xylose and cellobiose.

4.3 MATERIALS AND METHODS

Culture Maintenance

Clostridium beijerinckii NCIMB 8052 (ATCC number 51743) was obtained from TetraVitae Bioscience, Inc, Champaign, IL. The culture was first revived from the frozen spores in TYG medium (Tryptone 3 g/l, yeast extract 2 g/l and glucose 1 g/l), and transferred to the Modifed P2 medium with 10 mM glucose as the sole carbon source every week. Modified P2 medium was prepared as described in the previous studies (Baer et al., 1987). The medium was first desonicated for 30 minutes, and then flushed with high purity grade nitrogen for 10 minutes (both aqueous phase and headspace) to maintain strictly anaerobic. The medium was autoclaved at 121 ℃ for 20 minutes and aseptic culture techniques were used throughout. The culture was incubated at 37 ℃ (Hatch and Finneran, 2008).

Reduced Electron Shuttles

Anthraquinone-2,6-disulfonate (AQDS) was purchased from Sigma-Aldrich (Milwaukee, WI). To generate AH_2QDS, 5 mM AQDS in 30 mM sodium bicarbonate buffer were bubbled with 80:20 H_2:CO_2 for at least 1 hour in the presence of 100 g/L palladium-coated Aluminum catalyst (Sigma) and then incubated at 30 ℃ overnight . Reduced anthraquinone-2,6-disulfonate (AH_2QDS) stock solution was then bubbled with hydrogen-free, nitrogen gas and double filtered with 0.2 micron filter (PALL Acrodisc® syringe filter) into a sterile, evacuated pressure tube. Hydrogen was not present in the stock solution.

Analytical Methods

Headspace hydrogen was quantified by injecting 0.5 ml headspace sample into a GC (Shimadzu GC-14A) with a carbosieve S-II Spherical Carbon Column (Supelco, Bellefonte, PA) equipped with a Thermo Conductive Detector (SRI instrument Model 110). Nitrogen was used as the carrier gas; the temperature of the detector and oven was set as 150 and 50 °C, respectively. Cell growth was monitored by measuring optical density (OD) at 600 nm (Hatch and Finneran, 2008). Aqueous samples were collected with sterilized syringes and filtered through 0.2 micron sterile PTFE syringe filters. Carbohydrate concentration was quantified by phenol-sulfuric method in P2 medium (Taylor, 1995).

Batch Growth Experiments

Experiments with growing cells were conducted in anaerobic test tubes with a fixed volume (total volume of 16 ml). Modified P2 medium was used at different AH_2QDS concentrations and substrate conditions to study the influence of reduced electron shuttles on hydrogen production kinetics. The initial substrate concentration was 2 g/l for glucose, xylose and cellobiose, respectively, for experiments that investigated the influence of different AH_2QDS concentrations on hydrogen production. Substrate concentration varied at 1, 2 and 5 g/L for glucose, xylose and cellobiose, respectively, to investigate the influence of hydroquinone on hydrogen kinetics at different substrate concentration. Headspace and aqueous samples were collected routinely using anoxic, sterile syringes and needles. All batch tests were run in triplicate.

Resting Cell Suspension Experiment

Resting cell suspensions were performed to quantify hydrogen production rates under non-growth conditions in anaerobic phosphate buffer (0.5g/L potassium phosphate buffer) (Hatch and

Finneran, 2008). *C. beijerinckii* cells were harvested in late log phase of growth. One liter of cells was harvested with a heavy sterilized N_2 flow, and centrifuged and washed twice at 5000g for 15 min to form a dense cell pellet. The pellet was resuspended in 1.0 mL of 0.5 g/L anaerobic phosphate buffer and sealed immediately under a pure sterilized N_2 headspace. The experiments were initiated by inoculating the resuspended pellets into the various experimental tubes. Experimental tubes contained 0.2 mL of cells in a total volume of 10 mL of phosphate buffer with an anaerobic N_2 headspace. Experimental tubes were incubated with 250-500 µM AH_2QDS in the presence of 200 µM Glyceraldehyde-3-Phosphate. Control tubes were cells alone and cells plus 200 µM Glyceraldehyde-3-Phosphate without AH_2QDS addition. All experiments were run in triplicate and tubes were incubated at 37 ºC.

Kinetic Modeling of Hydrogen Production, Substrate Utilization and Cell Growth

A modified Gompertz equation (Equation. 4.1) was used to fit the cumulative hydrogen production and growth curve for each batch test to obtain the maximum product production $P_{max,i}$, product formation rate R_i, and lag phase λ_i, where i represents hydrogen and cell density, respectively (Lin and Lay, 2004; Mu et al., 2006; Zwietering et al., 1990). The same equation was also used to fit the substrate utilization, plotted as substrate utilized versus time.

$$P = P_{,} \exp\left(-\exp\left(\frac{}{_{,}}(\lambda - t) + 1\right)\right) \qquad \text{(Equation 4.1)}$$

$P_{hydrogen}$ and $P_{max,hydrogen}$ are expressed as µmole and $R_{hydrogen}$ is reported as µmole*h^{-1}. The hydrogen production rate $R_{hydrogen}$ was normalized by the volume of the medium to obtain the specific hydrogen production rate in units of mmole*L^{-1}*h^{-1}. $P_{max,\ growth}$ and P_{growth} are the optical density data, as a result unitless; and R_{growth} is in units of h^{-1}. λ_i is in units of hours for all the products.

4.4 RESULTS AND DISCUSSION

Influence Of AH_2QDS Addition On Hydrogen Production Kinetics From Glucose

Batch hydrogen production experiments were conducted in anaerobic tubes with fixed headspace volume. AH_2QDS at concentrations ranging from 100 μM to 500 μM was amended to batch fermentation cultures to investigate its influence on hydrogen production and cellular growth, with no AH_2QDS and 500 μM AQDS amendments as controls.

Figure 4.1 shows the cumulative hydrogen production and biomass growth as a function of time at different AH_2QDS concentration. Hydrogen started to accumulate after a lag phase of approximately 5 hours. When reduced electron shuttles were present at concentrations of 100 to 500 μM, both hydrogen and cell density reached maximum around 24 hours after the inoculation. Cumulative hydrogen was in the range of 285-295 μmole per test tube and cell density (OD 600) was 0.83-0.85. For cell alone and oxidized electron shuttle amended control experiments, cumulative hydrogen was 221-230 μmole per test tube at 24 hours after the inoculation, which was about 75% of the hydrogen production when AH_2QDS was added. Even though adding reduced electron shuttles significantly increased the hydrogen production at 24 hours, it did not change the overall hydrogen production at the end of the batch experiments, and the cumulative hydrogen production was in the range of 276-300 μmole per test tube, irrespective of AH_2QDS addition.

Adding 100-500 μM AH_2QDS increased specific hydrogen production rate by 81-99% relative to unamended control, while the oxidized form of electron shuttles (AQDS) did not affect the specific hydrogen production rate (Figure 4.2). Adding reduced electron shuttles also improved the apparent growth rate and substrate utilization rate by approximately 124% times

and 91%, respectively. The increase of specific hydrogen production was consistent with the increase of apparent growth rate and substrate utilization rate, which suggested that adding reduced electron shuttles (AH_2QDS) might influence the global cellular metabolism to increase the hydrogen production rate, other than selectively stimulate the hydrogen production pathways.

This is the first study to our knowledge to quantitatively show that adding reduced electron shuttling compounds stimulated hydrogen production kinetics. Reduced electron shuttling compounds have been suggested to stimulate the substrate utilization and product formation rate in previous studies (Park and Zeikus, 1999). Park and Zeikus demonstrated that reduced neutral red significantly improved succinate production and growth rate, and stimulated the glucose utilization in pure culture *A. succinogenes* system. They suggested that extracellular electron shuttling compounds might be able to control the intracellular $NADH/NAD^+$ ratio, replace the function of menaquinone in the fumarate reductase complex, and affect the membrane-bound enzymatic reactions (Park and Zeikus, 1999). AH_2QDS has a similar chemical structure of neutral red and can possibly affect the intracellular electron transport chain by serving as an artificial electron carrier.

Influence of AH_2QDS Addition On Hydrogen Production At Different Glucose Concentrations

Batch experiments were conducted to study the influence of AH_2QDS addition on hydrogen production at different glucose concentration. The AH_2QDS addition was fixed at 250 μM; the initial substrate concentrations varied at 1, 2 and 5 g/l.

Table 4.1 summarizes the specific hydrogen production rate, maximum hydrogen production and hydrogen molar yield at different glucose concentration. Adding AH_2QDS increased the specific hydrogen production rate by 110, 78 and 40% at initial substrate concentrations of 1, 2 and 5 g/l, respectively. The increase was more significant at substrate concentration of 1 and 2

g/L (over 75% increase) compared to the increase at 5 g/L (approximate 40%). This was probably due to different hydrogen concentrations at different substrate concentration. At glucose concentrations of 1 and 2 g/L, hydrogen partial pressure in the closed batch system was below 45 kPa, while hydrogen partial pressure reached 65 kPa when glucose concentration was 5 g/L. It is possible that high hydrogen partial pressure to some extent inhibited the biochemical hydrogen production pathway, which resulted in a smaller increase of specific hydrogen production rate at glucose concentration of 5 g/L. Adding reduced electron shuttles did not increase the hydrogen molar yield in the glucose experiment. Maximum hydrogen molar yield was observed when the substrate concentration was 2 g/l, which was the medium range of all the tested conditions. This trend also coincided with several previous studies. Van Ginkel et al. demonstrated that 7.5 g COD/l substrates resulted in the maximum hydrogen yield (tested range: 1.5-44.8 g/l) in a mixed culture system (Ginkel et al., 2001). Xing et al. supported that a medium range of substrate concentration was optimal for a maximum hydrogen yield of 1.93 mole/mole glucose in a pure culture *Enterobacter* system (Xing et al., 2008). The substrate insufficiency at low substrate concentration and the hydrogen partial pressure inhibition at high substrate concentration are the possible reasons for the optimal substrate concentration at the medium range (Kargi and Pamukoglu, 2009).

Influence of AH_2QDS addition on hydrogen production kinetics from xylose and cellobiose

Figure 4.3 shows the influence of AH_2QDS addition on specific hydrogen production rate, apparent growth rate, and substrate utilization rate when xylose (Figure 4.3 (a)) and cellobiose (Figure 4.3 (b)) were the primary substrate, respectively.

Adding reduced electron shuttles (AH_2QDS) from 100 to 500 μM also improved the kinetics of the fermentative hydrogen production process in the batch experiments when xylose

and cellobiose were the sole substrate, respectively. When xylose was the primary substrate, adding 100 μM AH_2QDS increased the specific hydrogen production rate by 40%; a further increase of AH_2QDS concentration to 250-500 μM resulted in a continuous increase of over 1-fold. Similarly, when cellobiose was the primary substrate, adding 250-500 μM AH_2QDS increased the hydrogen production rate by over 54%.

As observed in the glucose experiment, the increase of specific hydrogen production rate was consistent with the increase of substrate utilization rate and apparent growth rate in both xylose and cellobiose experiments, which supported the previous hypothesis that the influence of reduced electron shuttles on hydrogen production rate was probably due to the global influence of AH_2QDS on the cellular metabolism.

The apparent growth rate of the cellobiose experiment was lower than that when xylose was the primary substrate, but the specific hydrogen production rate was slightly higher than that of xylose experiment. Cellobiose, a disaccharide, consists of two D-glucopyranoses joined by a 1,4',-beta-glycoside bond (Ohdan et al., 2007). Limited studies have been done to investigate the mechanism of how cellobiose is assimilated in *Clostridium*; however, current knowledge suggests that 1 mole cellobiose is either directly cleaved into 2 mole glucose by β-glycosidase or converted to 1 mole Glucose-1-phosphate and 1 mole glucose by cellobiose phoshporylase (Mitchell et al., 1995; Ohdan et al., 2007). Both mechanisms require extra steps to convert cellobiose into glycolysis; thus it is reasonable that the substrate utilization rate and growth rate from cellobiose was lower than that of xylose experiment. However, one mole of cellobiose is converted into 2 mole monosaccharides, which theoretically doubles the hydrogen production. As a result, the specific hydrogen production rate of cellobiose experiment surpassed the rate when xylose was the primary substrate.

Influence of AH_2QDS Addition On Hydrogen Production At Different Xylose and Cellobiose Concentrations

Table 4.2 summarizes the specific hydrogen production rate, apparent growth rate, and substrate utilization rate at different xylose and cellobiose concentrations plus and minus AH_2QDS addition. Adding 250 μM AH2QDS increased the specific hydrogen production rate by 1.8, 1.0 and 2 fold at xylose concentration of 1, 2 and 5 g/L, respectively. The increase of hydrogen production rate was consistent with the improvement of cellular growth rate and substrate utilization rate. When cellobiose was the primary substrate, adding AH_2QDS increased the specific hydrogen production rate by 90, 79 and 70% when the substrate concentration was at 1, 2 and 5 g/L, respectively. Even though adding AH_2QDS significantly increased the hydrogen production rate in both xylose and cellobiose experiments, it is interesting to notice that increase in xylose was higher than the increase in cellobiose experiment. *Clostridium beijerinckii* ferments xylose via the pentose phosphate pathway, while assimilates cellobiose via Embden-Meyerhof pathway besides an extra step to convert it to monosaccharide. The differences of the biochemical pathways and different electron transport systems might contribute to the different level of hydrogen production rate increase.

In xylose and cellobiose experiments, specific hydrogen production rate was similar irrespective of initial substrate concentration (0.6-0.8 and 1.08-1.34 mmole/L-h for xylose and cellobiose concentration at 1-5 g/L, respectively). This finding is consistent with previous reported results on how initial substrate concentration affected hydrogen production rate (Ren et al., 2009b; Zhao et al., 2010). Zhao et al. demonstrated that hydrogen production rate stayed almost constant at the corncob concentration of 15-30 g/L (Zhao et al., 2010). Ren et al. also

showed that hydrogen production rate remained within the range of 248.7–269.9 ml/l/h at different xylose concentration (Ren et al., 2009b).

Influence Of AH_2QDS Addition On The Hydrogen Production Rate From Glyceraldehydes-3-Phosphate (G3P)

Adding reduced electron shuttles universally increased the kinetics of hydrogen production and cellular growth from glucose, xylose and cellobiose; however, the biochemical pathways for *C.* beijerinckii to assimilate glucose, xylose and cellobiose are not the same. For the 5-carbon sugar (xylose), the substrate was metabolized via pentose phosphate pathway to glyceraldehyde-3-P (G3P). As to glucose and cellobiose, the substrates were assimilated via glycolysis to glyceraldehyde-3-P (G3P). Thus, we hypothesized that adding reduced electron shuttles increased the evolution rate from glyceraldehyde-3-P (G3P), thereby improving the kinetics for hydrogen production. Cell suspension experiments were conducted to study the influence of reduced electron shuttles on hydrogen evolution rate with glyceraldehyde-3-P as the sole substrate. Resting cell experiments were necessary to quantify the influence of AH_2QDS on hydrogen evolution rate from glyceraldehyde-3-P, as G3P is an intermediate of the sugar metabolism and cell suspension ceased the growth of the culture and continuous production of intracellular G3P.

Figure 4.4 shows the specific hydrogen production rates at different AH_2QDS concentrations. When AH_2QDS was not present, the mean specific hydrogen production rate from three independent experiments was 0.14 mmole/L-h. Adding 250-500 μM AH_2QDS increased the mean specific hydrogen production rate to 0.19 and 0.20 mmol-H_2/L-h. This data suggested that adding AH_2QDS possibly increased the hydrogen production rate from glyceraldehyde-3-P. However, the increase of specific hydrogen production rate was more

significantly increased in the growth experiment (over 1-fold increase) compared to the resting cell suspension experiments (36-54% increase). In the growth experiment, glyceraldehyde-3-P was produced intracellularly, which can be directly utilized by glyceraldehyde-3-P dehydrogenase to produce D-glycerate 1,3-bisphosphate. The cross-membrane transport of glyceraldehyde-3-P might decrease the efficiency of the overall process compared to the growth experiment.

4.5 CONCLUSION

We demonstrated successfully that adding reduced electron shuttles (anthrahydroquinone disulfonate; AH_2QDS) at low concentration increased the hydrogen production rate in *C. beijerinckii* fermentation from glucose, xylose and cellobiose, three major carbohydrates from the pretreatment of lignocellulosic biomass. Adding 100-500 μM AH_2QDS increased the specific hydrogen production rate, apparent growth rate and substrate utilization rate by 99%, 124% and 91%, respectively when glucose was the primary substrates. Adding 250 μM AH_2QDS increased the specific hydrogen production rate by 110%, 78% and 40% when glucose concentration was 1, 2 and 5 g/L, respectively. When xylose and cellobiose were the primary substrates, adding 100-500 μM AH_2QDS increased the specific hydrogen production rate by around 1-fold and 56%, respectively. The consistency of the rate increase among hydrogen production, biomass growth and substrate utilization suggested that AH_2QDS globally influenced the cellular metabolism. Resting cell suspension experiments showed an average 36-54% increase of specific hydrogen production rate from glyceraldehyde-3-Phosphate was observed when 250 and 500 μM AH_2QDS was added under non-growth, resting cell conditions. The increases of specific hydrogen production may ultimately decrease the reactor size for potential continuous hydrogen production at industrial scale.

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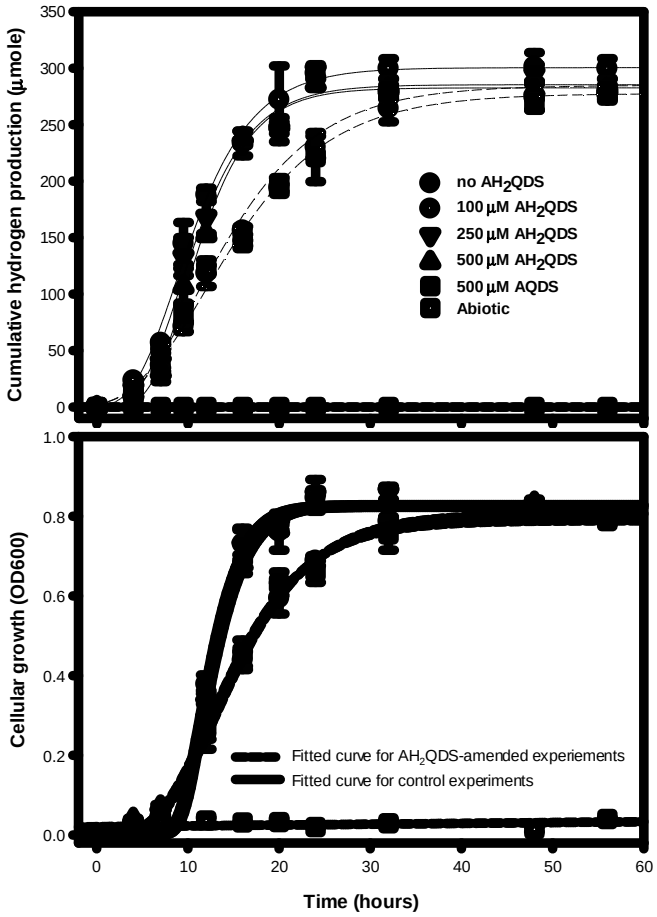

Figure 4.1 Cumulative hydrogen production (a) and growth (b) as a function of time at different AH_2QDS concentration. Data points are the means of triplicate analyses. Bars indicate one standard deviation. Lines are the fitted results from Modified Gompertz equation (Equation 4.1).

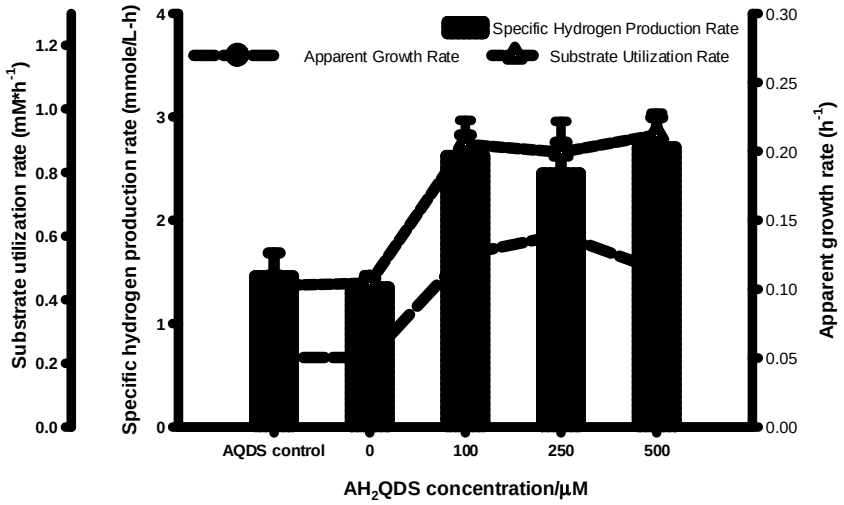

Figure 4.2 Specific hydrogen production rates, apparent growth rates, and substrate utilization rates at different AH_2QDS concentrations. Data points are the means of triplicate analyses and modeling parameters. Bars indicate one standard deviation.

Table 4.1 Summary of specific hydrogen production rate, maximum hydrogen production and hydrogen molar yield plus (+)/minus (-) AH_2QDS addition at different glucose concentrations

Substrate concentration	1 g/L		2 g/L		5 g/L	
AH_2QDS Amendment	(-)	(+)	(-)	(+)	(-)	(+)
Specific hydrogen production rate (mmole/h-L)	1.2±.2	3.0±.3	1.4±.1	2.5±.3	4.3±.4	6.2±.9
Maximum hydrogen production (μmole)	101±3	115±9	277±6	285±7	428±1	430±14
Hydrogen molar yield	1.8±.1	2.1±.2	2.5±.1	2.6±.1	1.5±.0	1.6±.1

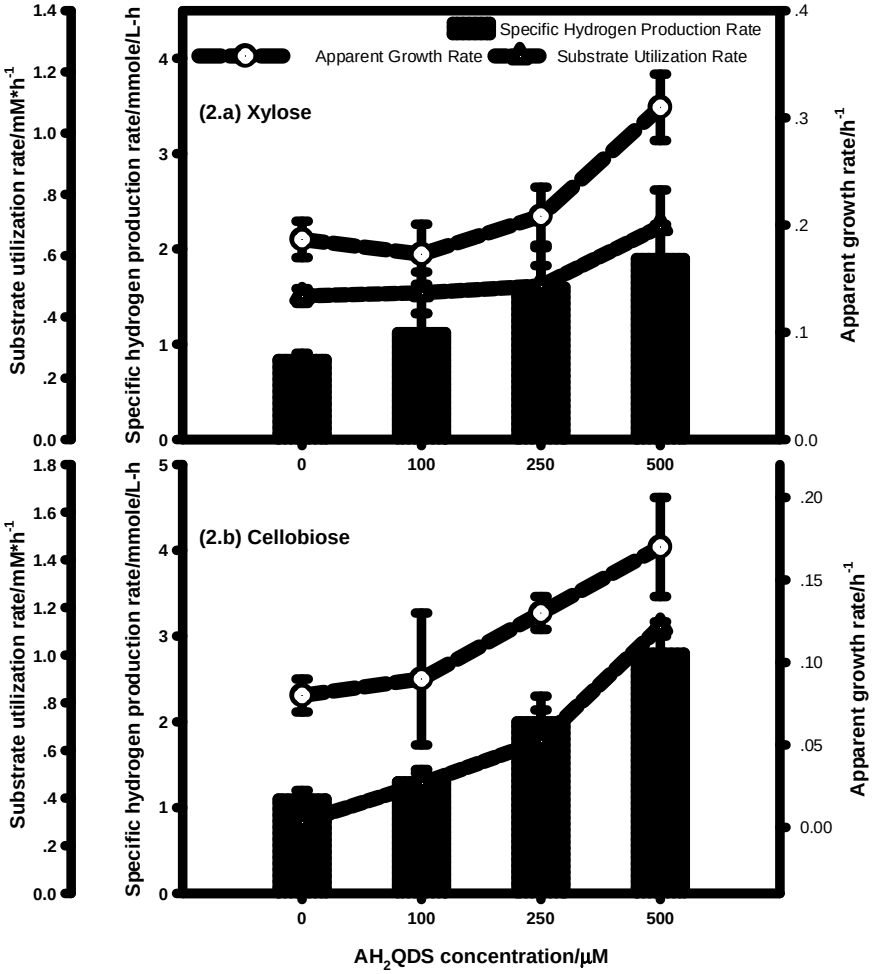

Figure 4.3 Specific hydrogen production rate, apparent growth rate and substrate utilization rate at different AH_2QDS concentrations. Data points are the means of triplicate analyses and modeling parameters. Bars indicate one standard deviation.

Table 4.2 Summary of specific hydrogen production rate, apparent growth rate and substrate utilization rate plus/minus AH_2QDS addition at different xylose and cellobiose concentrations

	Substrate concentration	1 g/l		2 g/l		5 g/l	
	AH_2QDS Amendment	(-)	(+)	(-)	(+)	(-)	(+)
Xylose	Specific hydrogen production rate (mmole/L-h)	0.6 (±.06)	1.7 (±.1)	0.8 (±.07)	1.6 (±.2)	0.6 (±0.09)	1.8 (±.2)
	Apparent growth rate (1/h)	0.16 (±0.01)	0.37 (±0.1)	.19 (±.02)	.21 (±.03)	0.18 (±0.01)	0.34 (±0.05)
	Substrate utilization rate (mM/h)	0.40 (±0.06)	0.79 (±0.26)	.47 (±0.02)	0.50 (±0.13)	0.59 (±0.18)	0.89 (±0.33)
Cellobiose	Specific hydrogen production rate (mmole/L-h)	1.08 (±0.09)	2.12 (±.28)	1.10 (±.12)	1.97 (±.19)	1.34 (±.17)	2.29 (±.15)
	Apparent growth rate (1/h)	0.09 (±0.01)	0.12 (±0.04)	0.07 (±0.01)	0.13 (±0.01)	0.13 (±0.02)	0.17 (±0.01)
	Substrate utilization rate (mM/h)	0.27 (±0.03)	0.41 (±0.02)	0.30 (±0.03)	0.74 (±0.13)	0.47 (±0.12)	0.60 (±0.09)

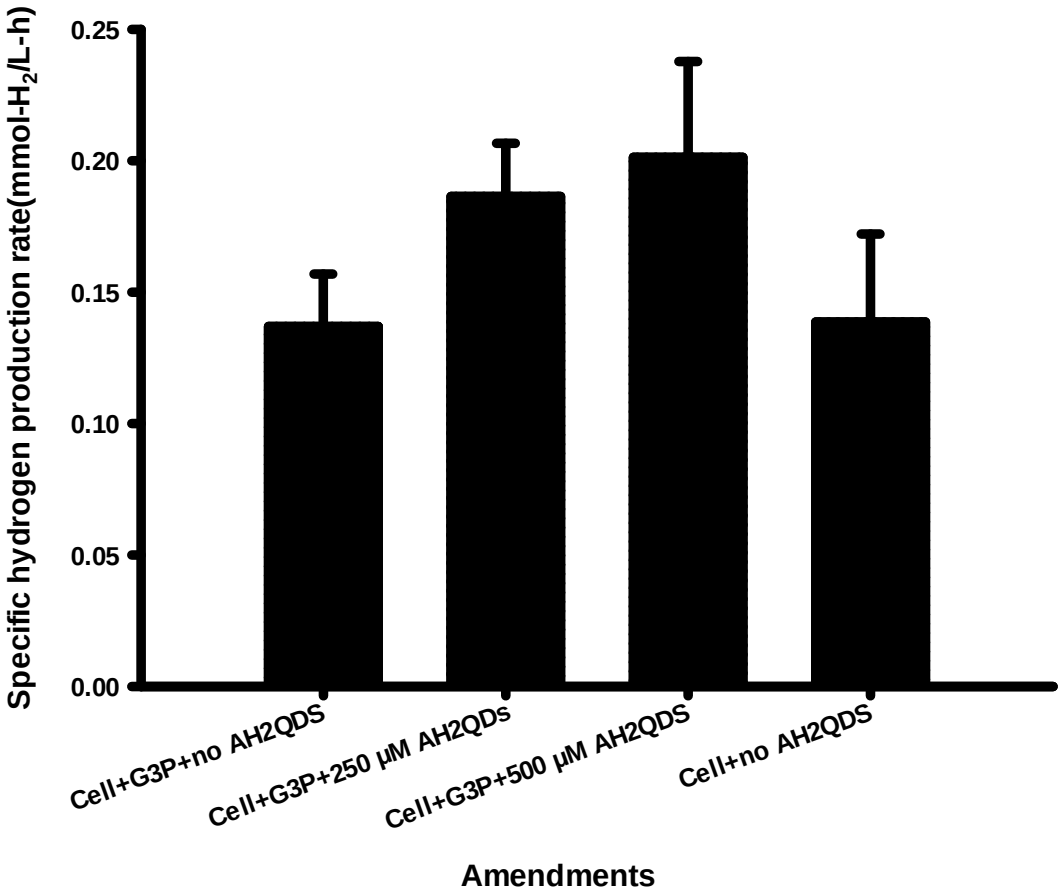

Figure 4.4 Specific hydrogen production rates at different amendments under resting cell conditions. Data points are the means of specific hydrogen production rates from Modified Gompertz equation (Equation 4.1). Bars indicate one standard deviation.

CHAPTER 5 REDUCED ELECTRON SHUTTLES INCREASE XYLOSE UTILIZATION AND HYDROGEN PRODUCTION IN MIXED SUGAR FERMENTATION

5.1 ABSTRACT

The influences of extracellular reduced electron shuttle (anthrahydroquinone-2,6-disulfonate, AH_2QDS) addition on substrate utilization and hydrogen production were investigated at different glucose:xylose ratios using the fermentative culture *Clostridium beijerinckii*. 250 μM extracellular hydroquinone addition increased the total substrate utilization by 23-66%, xylose utilization by 20-54% and hydrogen production by 15-54% at glucose:xylose ratios of 1:1, 1:3 and 1:9. Adding 250 μM AH_2QDS also increased the kinetics of hydrogen production by 44-94% and substrate utilization by 40-88% at all tested glucose:xylose ratios from 1:9 to 9:1. Increasing AH_2QDS concentrations from 250 μM to 2mM increased xylose utilization and the cumulative hydrogen production in the mixed sugar fermentation. The influence of glucose:xylose ratios on the hydrogen production kinetics and substrate utilization were also studied, and the increase of glucose:xylose ratios resulted in the increases of specific hydrogen production rates, substrate utilization rates, and the extent of substrate utilization in batch *Clostridium beijerinckii* fermentation. These data demonstrate that the substrate utilization, especially xylose utilization, can be manipulated by amending extracellular redox active compounds. This might impact the lignocellulosic based biofuel production by allowing us to develop physiological approaches to improve the pentose utilization in the fermentation of the hydrolysates of lignocellulosic materials.

Key words: Electron shuttles, AH_2QDS, xylose utilization, hydrogen, glucose:xylose ratio

5.2 INTRODUCTION

Lignocellulose such as agricultural residuals (e.g. corn stover and sugarcane bagasse), economic crops (e.g. switchgrass and miscanthus) and forest products (e.g. hardwood) is the most abundant renewable resource on the earth and can be used by industrial microorganisms after pretreatment and hydrolysis as substrates for fermentation (Jojima et al., 2010; Kumar et al., 2009). The principle components of lignocellulosic biomass include cellulose, hemicellulose, and lignin, which are tightly bound by hydrogen and covalent bonds (Malherbe and Cloete, 2002). The chemical structure and the presence of lignin, which glues the cellulose and hemicellulose, result in the recalcitrant nature of lignocellulosic biomass to allow direct biological conversion (Himmel et al., 2007). Thus, various physicochemical (e.g. Steam explosion and ammonia fiber explosion) (Kim and Lee, 2005; Li et al., 2007), chemical (e.g. acid and alkaline hydrolysis) (Esteghlalian et al., 1999; Kaar and Holtzapple, 2000) and biological methods (e.g. white-rot fungus *P. chrysosprium*) (Hatakka, 1983) have been investigated to break down the chemical structure to make cellulose and hemicellulose accessible to hydrolysis and saccharification. The lignocellulosic hydrolysates, which contain both hexose and pentose monomers, can be further converted to desired energy carriers in the fermentation process.

Glucose, the major building block of cellulose is considered as the most abundant sugar from the treatment of lignocellulose. Glucose can be readily fermented to bioenergy carriers by many industrial microorganisms (e.g. *Saccharomyces cerevisiae* and *Clostridium beijerinckii*) (Ezeji et al., 2007; Jojima et al., 2010). However, xylose, the major building block of hemicellulose and the second most abundant sugar from

lignocellulose cannot be efficient converted by most industrial microorganisms (Saha, 2003; Temudo et al., 2009). It is important to increase the utilization of xylose in the mixed sugar fermentation process. Several approaches involving genetic engineering have been proposed to increase xylose utilization in yeast and other industrial microorganisms (Gu et al., 2009; Young et al., 2010).

Due to the complex nature and different cellulose and hemicellulose composition in lignocellulosic feedstock, the glucose and xylose content in the hydrolysates varied significantly depending on the pretreatment and hydrolysis methods (Agbogbo et al., 2006). Table 5.1 summaries the calculated glucose:xylose ratio from different pretreatment and hydrolysis of various lignocellulosic biomass. In the downstream fermentation process, the glucose:xylose ratio has been investigated to influence the substrate utilization and product formation (i.e. bioethanol and biohydrogen). Zhao et al. also suggested that glucose:xylose ratio influenced the cell growth, substrate utilization and ethanol yield in pure culture *Pachysolen tannophilus* fermentation (Zhao et al., 2008). The glucose:xylose ratio has also been statistically shown to be a critical factor for efficient fermentative hydrogen production (Prakasham et al., 2010).

Electron shuttles are the group of redox active molecules that has been widely investigated to facilitate the electron transfer from electron donors to acceptors in biodegradation (Hernandez and Newman, 2001; Lovley et al., 1996; Lovley et al., 1999; Roden et al., 2010; Schwarzenbach et al., 1990). A few studies have investigated its influence on the fermentative physiology. Hatch and Finneran showed an increase of hydrogen production as a result of reduced electron shuttles addition in resting cell

suspensions of *C. beijerinckii.* Girbal et al. demonstrated the influence of neutral red on the product formation of *C. acetobutylicum* fermentation (Girbal et al., 1995).

In this research, we studied the hydrogen production and substrate utilization in mixed sugar (glucose and xylose) fermentation system using pure culture *Clostridium beijerinckii* NCIMB 8052, which is a fermentative, hydrogen-producing microorganism that uses several substrates, including both glucose and xylose (Lin et al., 2007). In this study, we investigated the influence of reduced electron shuttles (anthrahydroquinone disulfonate, AH_2QDS) on the substrate utilization and hydrogen production at different glucose:xylose ratios.

5.3 MATERIALS AND METHODS

Culture Maintenance

Clostridium beijerinckii NCIMB 8052 (ATCC number 51743) was obtained from TetraVitae Bioscience, Inc, Champaign, IL. The culture was revived from frozen spores in TYG medium (Tryptone 3 g/l, yeast extract 2 g/l and glucose 1 g/l), and was transferred to Modified P2 medium each week (Baer et al., 1987). Standard anaerobic and aseptic culturing techniques were used throughout. Media were sparged with N_2 passed over a heated, reduced copper column to remove trace oxygen from the gas line. Headspaces were flushed and sealed under N_2 headspace using a butyl rubber stopper fastened with an aluminum crimp. All subsequent amendments or transfers were made using sterile needles and syringes that had been flushed with anaerobic gas. Cultures were incubated at 37 °C (LabLine Imperial III Incubator).

Batch Experiments

Cells were grown in anoxic pressure tubes with fixed aqueous and headspace volumes. Total sugar concentration was fixed at 2 g/L and glucose:xylose ratio varied at 1:9, 1:3, 1:1, 3:1 and 9:1. Modified P2 medium (Baer et al., 1987) was used with fixed AH_2QDS concentration (250 µM) at different glucose:xylose ratios to investigate the pattern of substrate utilization and the influence of AH_2QDS on the substrate utilization and hydrogen production from mixed sugar. AH_2QDS amendments were scaled from 250 µM to 2 mM to study the influence of AH_2QDS concentration on substrate utilization and hydrogen production at fixed glucose:xylose ratio of 1:1. Headspace and aqueous samples were collected using anoxic, sterile syringes and needles. Tubes were incubated at 37 °C, without shaking, in the dark. All batch testes were run in triplicate.

Chemicals

Anthraquinone-2,6-disulfonate (AQDS) was purchased from Sigma-Aldrich (Milwaukee, WI). To generate AH_2QDS, 5 mM AQDS in 30 mM sodium bicarbonate buffer were bubbled with 80:20 H_2:CO_2 for at least 1 hour in the presence of 100 g/L palladium-coated Aluminum catalyst (Sigma) and then incubated at 30 °C overnight. Reduced anthraquinone-2,6-disulfonate (AH_2QDS) stock solution was then bubbled with hydrogen-free, nitrogen gas and double filtered with 0.2 micron filter (PALL Acrodisc® syringe filter) into a sterile, evacuated pressure tube. Hydrogen was not present in the stock solution.

Analytical Techniques

0.5 ml gas headspace samples were collected and injected into a GC (Shimadzu GC-14A) with a carbosieve S-II Spherical Carbon Column (Supelco, Bellefonte, PA)

equipped with a Thermal Conductivity Detector. Nitrogen was the carrier gas; the temperatures of the detector and oven were set as 150 and 50 ℃, respectively.

Aqueous samples were collected with sterilized syringes and filtered through 0.2 micron sterile PTFE syringe filters. The samples were analyzed for carbohydrates (glucose and xylose) using an HPLC (Dionex Ultimate 300) system equipped with a Bio-Rad HPX-87H column (Sluiter, 2008). 5 mM sulfuric acid was the eluent; flow rate was 0.6 ml; column temperature was set at 55-65 ℃, and the detector temperature was 60 °C (Sluiter, 2008).

Kinetic Modeling

A modified Gompertz equation (Equation 5.1) was used to fit the cumulative hydrogen production curve to obtain the hydrogen production rate R_{H2} (Lin and Lay, 2004; Mu et al., 2006; Zwietering et al., 1990). Specific production rate (in units of mmole H_2/L-h) were calculated by normalizing the R_{H2} by the volume of the medium in the batch experiments. The same equation was also used to fit the substrate utilization, plotted as substrate utilized versus time. Substrate utilization rates are expressed as g/L-h.

$$P = P_{,} \exp\left(-\exp\left(\frac{}{_{,}}(\lambda - t)\right) + 1\right) \qquad \text{(Equation 5.1)}$$

5.4 RESULTS AND DISCUSSION

Reduced Electron Shuttles (AH_2QDS) Addition Increased Substrate Utilization And Cumulative Hydrogen Production At Different Glucose:Xylose Ratio

Batch experiments were run at different glucose:xylose ratios with a total initial sugar concentration of 2 g/L. AH_2QDS concentration was fixed at 250 µM with non-amended as control. Figure 5.1 shows the cumulative hydrogen production and total substrate utilization at different glucose:xylose ratio plus/minus AH_2QDS addition. Adding 250 µM AH_2QDS increased the total substrate utilization and cumulative hydrogen production at glucose:xylose ratio of 1:9 and 1:3. At glucose:xylose ratio of 1:9, 72 µmole hydrogen was produced and 54% of total substrate was utilized at the end of fermentation process. Adding 250 µM AH_2QDS increased the hydrogen production to 104 µmole per test tube and the substrate utilization from 54% to 79%. When initial glucose:xylose ratio was set at 1:3, adding 250 µM AH_2QDS improved the substrate utilization from 66% to 81%, and enhanced the hydrogen production from 77 µmole to 116 µmole per test tube. When the glucose:xylose ratio was at 3:1 and 9:1, approximately 80-83% and 92-94% of the initial substrates were consumed and cumulative hydrogen production reached 103-109 and 118-123 µmole per test tube at the end of fermentation process, irrespective of AH_2QDS addition at initial glucose:xylose ratios of 3:1 and 9:1, respectively. Glucose was not detected at the end of fermentation process at all tested glucose:xylose ratio irrespective of AH_2QDS addition; however, adding AH_2QDS decreased the residual xylose concentration when glucose:xylose ratios were at 1:3 and 1:9 (Figure 5.2). At the glucose:xylose ratio of 1:9, 1.0 g/L xylose was left in the medium at the end of fermentation process and adding 250 µM AH_2QDS decreased the

residual xylose to 0.4 g/L. When the glucose:xylose ratio was 1:3, adding 250 µM AH_2QDS decreased the residual xylose from 0.8 to 0.5 g/L. When glucose was dominant in the mixed substrates (glucose:xylose ratios of 3:1 and 9:1), little xylose was consumed at the end of fermentation process, and adding AH_2QDS did not affect the residual xylose concentration.

Adding reduced electron shuttles increased the total substrate utilization and hydrogen production at glucose:xylose ratios of 1:9 and 1:3. The increase of total substrate utilization was due to the increase of xylose utilization, as the glucose was completely consumed in the batch fermentation. Glucose in general is considered a better substrate than xylose for fermentative microorganisms (Jeffries, 1983; Temudo et al., 2009); however, to our knowledge there is little data available on mixed glucose and xylose utilization in *C. beijerinckii* fermentation. The results from this study showed the pattern of glucose and xylose utilization in *C. beijerinckii* fermentation. The microorganism was able to utilize more substrates as the glucose:xylose ratio increased and the increase of substrate utilization resulted in the increase of hydrogen production. When glucose was dominant in the mixed sugar (glucose:xylose ratios of 3:1 and 9:1), around 30% xylose was fermented. When the glucose:xylose ratio was lower at 1:9 and 1:3, approximately 51 % xylose was utilized, but the total substrate utilization was 20-40% lower than that at glucose:xylose ratios of 3:1 and 9:1. Adding 250 µM AH_2QDS increased the xylose utilization by 43-54% and the total substrate utilization by 23-46%. The total substrate utilization at the presence of 250 µM AH_2QDS reached 79-81%, which were similar to the total substrate utilization at glucose:xylose ratio of 3:1 (Figure 5.1). This finding suggests that adding reduced electron shuttles might be an effective

strategy to improve the utilization of xylose-rich hydrolysates from lignocellulosic materials with high hemicellulose content (i.e. Rapeseed Straw and red maple in table 1) in *Clostridium* fermentation.

Increase Hydrogen Production And Substrate Utilization Kinetics By Adding AH_2QDS At Different Glucose:Xylose Ratios

Hydrogen started to accumulate after a lag time of around 10 hours at all tested conditions (Figure 5.3). At low glucose:xylose ratios (1:3 and 1:9), it took around approximately 32 hourly for hydrogen production to achieve its peak irrespective of AH_2QDS addition; however, the maximum hydrogen production with AH_2QDS addition was significantly higher than that without AH_2QDS addition. When the glucose:xylose ratio was relatively higher at 3:1 and 9:1, hydrogen production reached its maximum at approximately 18 and 24 hours after inoculation with and without AH_2QDS addition, respectively. Maximum hydrogen production remained almost constant irrespective of AH_2QDS addition.

Adding 250 µM AH_2QDS increased specific hydrogen production rates at all tested glucose:xylose ratio (Figure 5.4 (a)). Specific hydrogen production rates were 0.48, 0.53, 0.79 and 1.13 mmole H_2/L-h at glucose:xylose ratios 1:9, 1:3, 3:1 and 9:1, respectively, when AH_2QDS was not present in the growth condition. Adding 250 µM AH_2QDS increased the specific hydrogen production rate by 44-94% and the specific hydrogen production rates reached 0.85, 0.88, 1.54 and 1.63 mmole H_2/L-h at glucose:xylose ratios of 1:9, 1:3, 3:1 and 9:1, respectively. Reduced electron shuttles also increased the substrate utilization rates (Figure 5.4 (b)). At relatively low glucose:xylose

ratio of 1:9 and 1:3, adding 250 μM AH_2QDS increased the xylose utilization rates from 0.045 and 0.027 g/L-h to 0.073 and 0.046 g/L-h, respectively, while did not significantly affect the glucose utilization rate. When glucose:xylose ratios were at 3:1 and 9:1, adding 250 μM AH_2QDS increased the glucose utilization rates from 0.10 and 0.09 g/L-h to 0.14 and 0.17 g/L-h, respectively. Xylose utilization rates were less than 10% of the glucose utilization rates.

The increases of product formation rates were consistent with the increases of substrate utilization rates in the batch fermentation system. We previously demonstrated that adding reduced electron shuttles stimulated the substrate utilization rates and hydrogen production rates at single substrate system, and the extracellular reduced electron shuttles might increase the conversion rate of glyceraldehyde-3-Phosphate, which is a shared intermediate for both xylose and glucose metabolism, to pyruvate to enhance the substrate utilization rate. The increase of substrate utilization rate further resulted in the improvement of specific hydrogen production rate. In the mixed glucose and xylose fermentation system, at glucose:xylose ratios of 1:9 and 1:3, adding AH_2QDS increased the xylose utilization rates significantly but did not affect the glucose utilization rates. This was possibly due to the low glucose concentrations in the batch system. At glucose:xylose ratios of 3:1 and 9:1, adding reduced electron shuttles increased the glucose utilization rates and the xylose utilization was minimal irrespective of AH_2QDS additions. The presence and conversion of glucose possibly inhibited the xylose utilization, as previously reported in other fermentative microorganisms (Bertilsson et al., 2009; Kötter and Ciriacy, 1993; Meinander and Hahn-Hagerdal, 1997).

Increasing glucose:xylose ratios at the same total carbohydrates concentration increased the specific hydrogen production rates and substrate utilization rates (Figure 5.4), while concomitantly enhancing the overall hydrogen production and the extent of substrate utilization (Figure 5.1). Glucose has been reported to be a better substrate than xylose for many fermentative microorganisms (Jeffries, 1983; Temudo et al., 2009). *C. beijerinckii* can ferment both glucose and xylose to hydrogen and butanol, which makes it an industrially-important fermentative microorganism (Ezeji et al., 2007; Lin et al., 2007); however, the mechanism of glucose and xylose uptake and metabolism seems to be different in *Clostridium* fermentation (Mitchell, 1998). *C. beijerinckii* has been reported to take up glucose via a phosphoenolpyruvate-dependent phosphotransferase system (PTS), and xylose uptake in *C. beijerinckii* was reported to be unlikely PTS (Mitchell, 1996; Mitchell, 1998; Mitchell et al., 1995). The phosphotransferanse system is efficient, as there is no ATP consumption at the transport step (White, 2000). Little information is available on how xylose was transported into the cell in *C. beijerinckii*; it is likely that the xylose uptake consumes ATP (Temudo et al., 2009; Zhou et al., 2009). Moreover, glucose was metabolized by glycolysis and the ATP yield from the glycolysis was 2 mole ATP per mole glucose; xylose was metabolized by pentose phosphate pathway and 1.67 mole ATP was generated via substrate-level phosphorylation for every mole xylose fermented (Temudo et al., 2009; White, 2000). Xylose uptake might consume ATP, and the metabolism of xylose yields less ATP than that of glucose, which could very well explain the increase of substrate utilization rates and the extents of substrate utilization as the glucose:xylose ratio increased.

Influence Of AH_2QDS Concentration On Substrate Utilization And Hydrogen Production At Fixed Glucose:Xylose Ratio

A number of studies reported around a 1:1 ratio of glucose and xylose in the hydrolysates of the lignocellulosic materials (Hu et al., 2008; Kumar et al., 2009). Batch experiments were investigated at a fixed glucose:xylose ratio of 1:1 to study the influence of different AH_2QDS concentration on the substrate utilization and hydrogen production. Adding 250 µM to 2 mM AH_2QDS increased the total substrate utilization and hydrogen production. When AH_2QDS was not present, around 40-50% of total substrates were fermented to 87 µmole hydrogen (Figure 5.5 (a)). Adding 250 µM AH_2QDS increased the substrate utilization to 65% and cumulative hydrogen production to 100 µmole. Further increase of AH_2QDS concentration to 500 µM resulted in a corresponding increase of substrate utilization to 84% and the cumulative hydrogen production to 139 µmole. When AH_2QDS concentration reached 1 and 2 mM, almost all of the substrates were fermented and the cumulative hydrogen production reached 156 and 169 µmole.

Even though the total substrate utilization was only 40-50% in the *C. beijerinckii* fermentation system when AH_2QDS was not present, all the glucose was fermented, while almost none of the xylose was utilized (Figure 5.5 (b)). The increase of the total substrate utilization (Figure 5.5 (a)) was due to the decrease of the residual xylose concentration at the end of fermentation process, as all of the residual glucose concentration was below the detection limit of the carbohydrate analysis (Figure 5.b). At AH_2QDS concentration of 250 µM, around 0.5 g/L xylose were fermented along with the complete glucose consumption. The increase of AH_2QDS concentration further resulted in the increase of xylose utilization, and adding 500 µM, 1mM and 2mM AH_2QDS in the

growth medium resulted in an increase of xylose utilization from almost none (non-AH2QDS amended) to 76, 97 and 100%, respectively.

Electron shuttles have been reported to affect the metabolism of fermentative microorganisms. Hatch and Finneran reported the increase of hydrogen production with reduced electron shuttle addition in the resting cell suspension of *C. beijerinckii* (Hatch and Finneran, 2008). Since in the resting cell system it was not feasible to quantify the endogenous utilization of biomass as the substrates for hydrogen production, it was possible that adding reduced electron shuttles increased the endogenous substrate utilization to generate more hydrogen in the resting cell suspension system. Park et al. identified that the reduced electron shuttle (neutral red) could serve as the sole reducing power for the growth and metabolism of *Actinobacillus succinogenes* (Park et al., 1999). Extracellular electron shuttles have also been suggested to affect the concentrations and ratios of intracellular electron carriers (i.e. NADH and NAD^+) to influence product formation (Girbal et al., 1995; Peguin and Soucaille, 1995). The intracellular electron carrier profile has been investigated to affect the key enzymes of the substrate-level phosphorylation (Vasconcelos et al., 1994), which might be the link of the extracellular reduced electron shuttle addition and the increase of xylose utilization in this study.

5.5 CONCLUSION

This study provided evidence that the addition of reduced electron shuttles increased the xylose utilization and hydrogen production in mixed sugar fermentation. Adding 250 μM reduced electron shuttles (anthrahydroquinone-2,6-disulfonate, AH_2QDS) increased the total substrate utilization by 23-66%, xylose utilization by 20-54% and hydrogen production by 15-54% at glucose:xylose ratios of 1:1, 1:3 and 1:9, and the hydrogen production rate by 44-94% at all tested glucose:xylose ratios from 1:9 to 9:1. The increase of hydrogen production rates corresponded to the increases of substrate utilization rates. Increasing concentrations of AH_2QDS from 250 μM to 2mM increased the xylose utilization up to 100% at a glucose:xylose ratio of 1:1.

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Table 5.1 Different glucose:xylose ratios from the hydrolysis of lignocellulosic feedstocks (* glucose:xylose ratios were calculated from the literature)

Feedstock	Type of hydrolysis	glucose:xylose ratio*	Reference
Barley Straw	Acid-impregnation, steam explosion and enzymatic hydrolysis	5.6:1	(Rosgaard et al., 2007)
Corn stover	Lime and enzymatic hydrolysis	3:1	(Kim and Holtzapple, 2005)
Switchgrass	radio-frequency-assisted alkali and enzymatic hydrolysis	1.2:1	(Hu et al., 2008)
Red maple	Diluted sulfuric acid	1:2.2	(Yat et al., 2008)
Rapeseed Straw	Sulfuric acid	1:6.6	(Jeong et al., 2010)

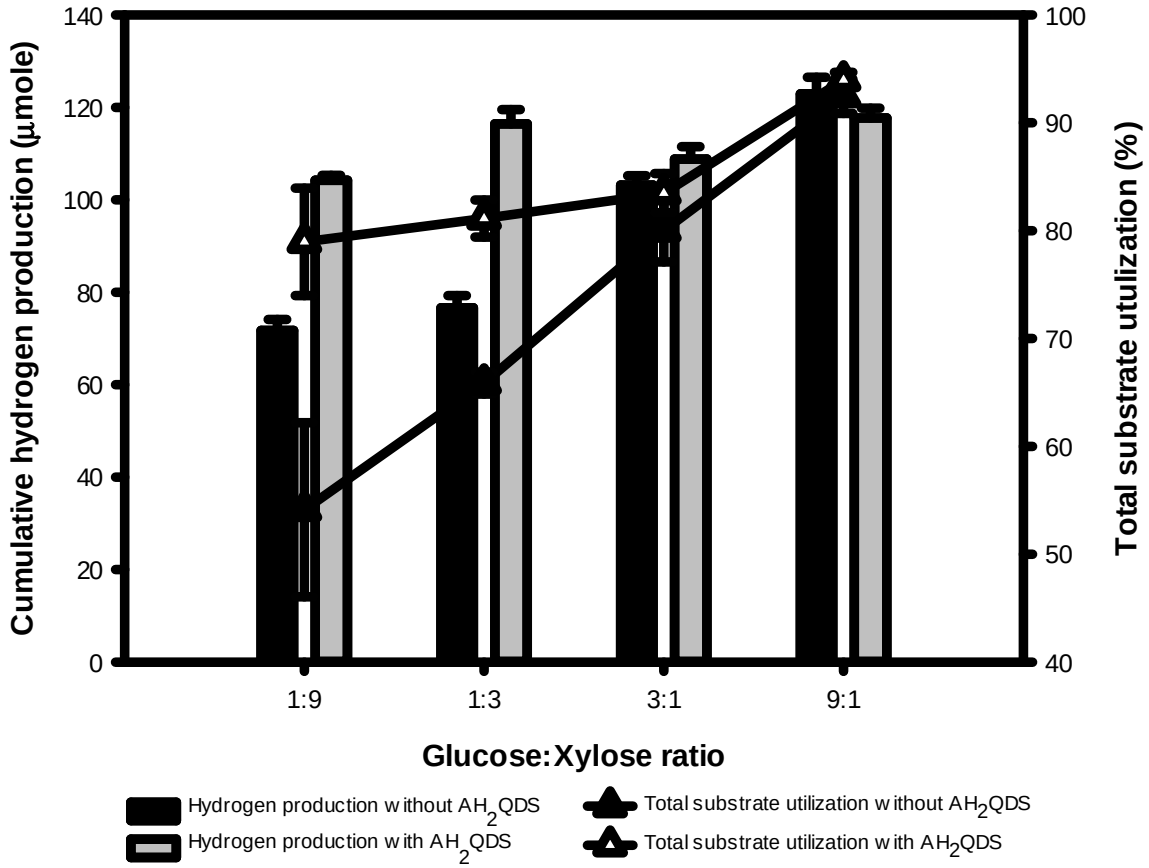

Figure 5.1 Cumulative hydrogen production and total substrate utilization at different glucose:xylose ratios with and without 250 µM AH_2QDS addition. Total substrate utilization was calculated as (glucose and xylose consumed)/(Initial glucose and xylose).

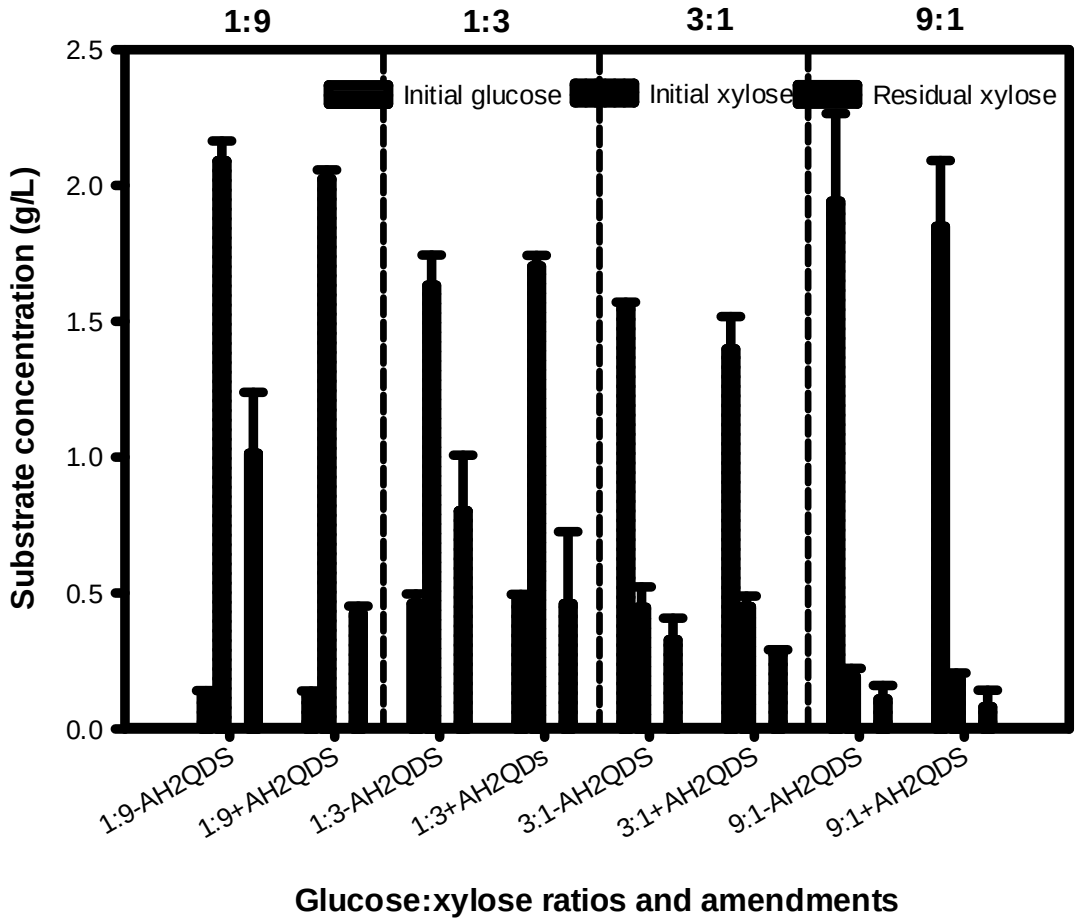

Figure 5.2 Initial and residual substrate concentrations in batch experiments at different glucose:xylose ratios with and without 250 μM AH_2QDS addition. Residual glucose concentrations were not shown as they were below the detection limit of the HPLC-RI analysis.

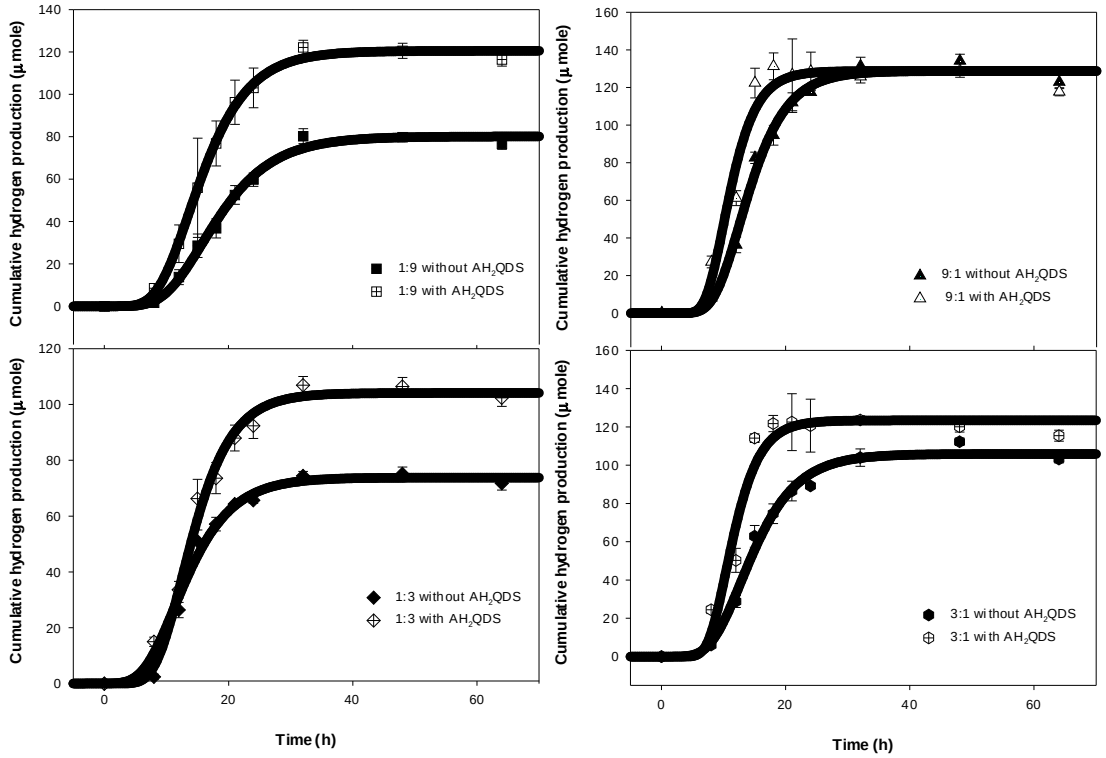

Figure 5.3 Cumulative hydrogen production as a function of time at different glucose:xylose ratios with and without 250 µM AH_2QDS addition. Data points are the means of triplicate analyses. Bars indicate one standard deviation. Lines are the fitted results from Modified Gompertz equation (Equation 5.1).

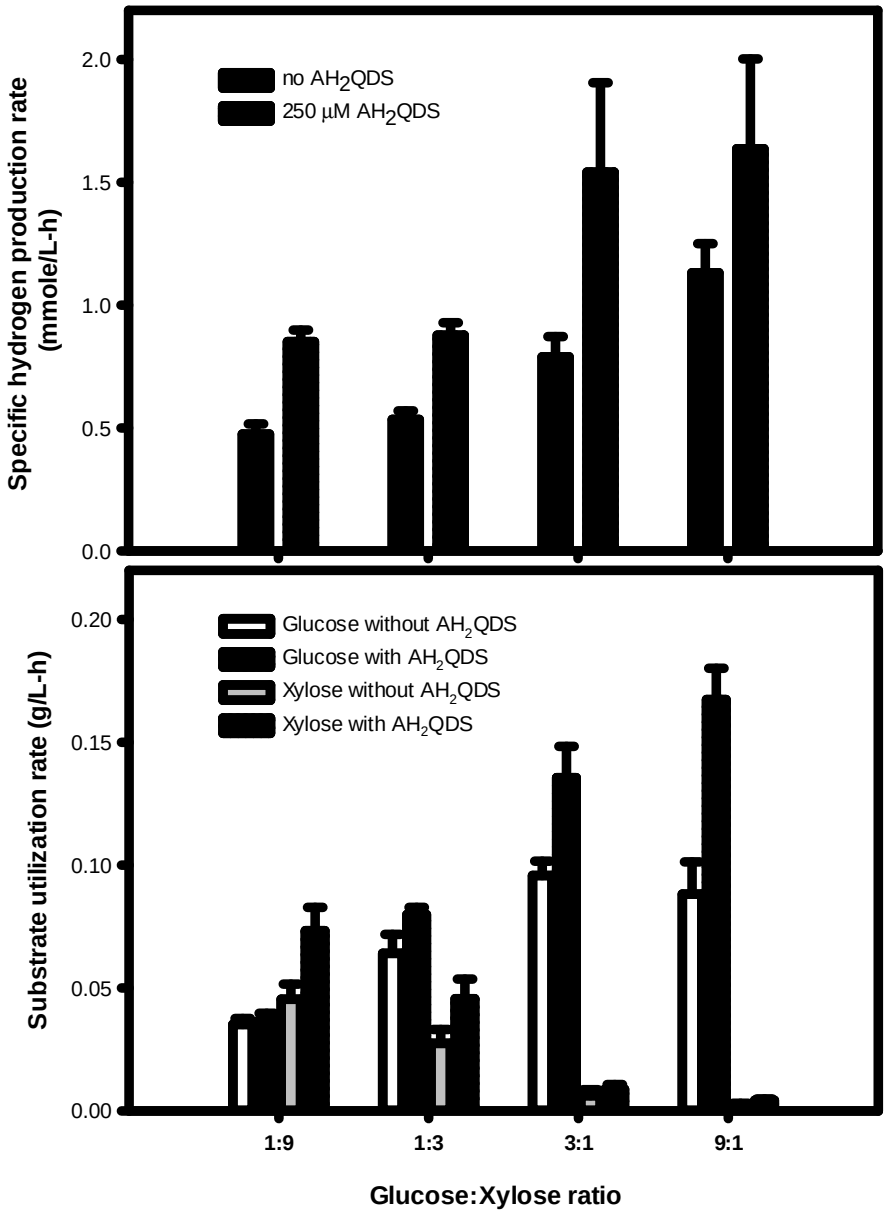

Figure 5.4 (a) Specific hydrogen production rate with and without 250 µM AH_2QDS addition at different glucose:xylose ratios. (b) glucose and xylose utilization rate with and without 250 µM AH_2QDS addition at different glucose:xylose ratios.

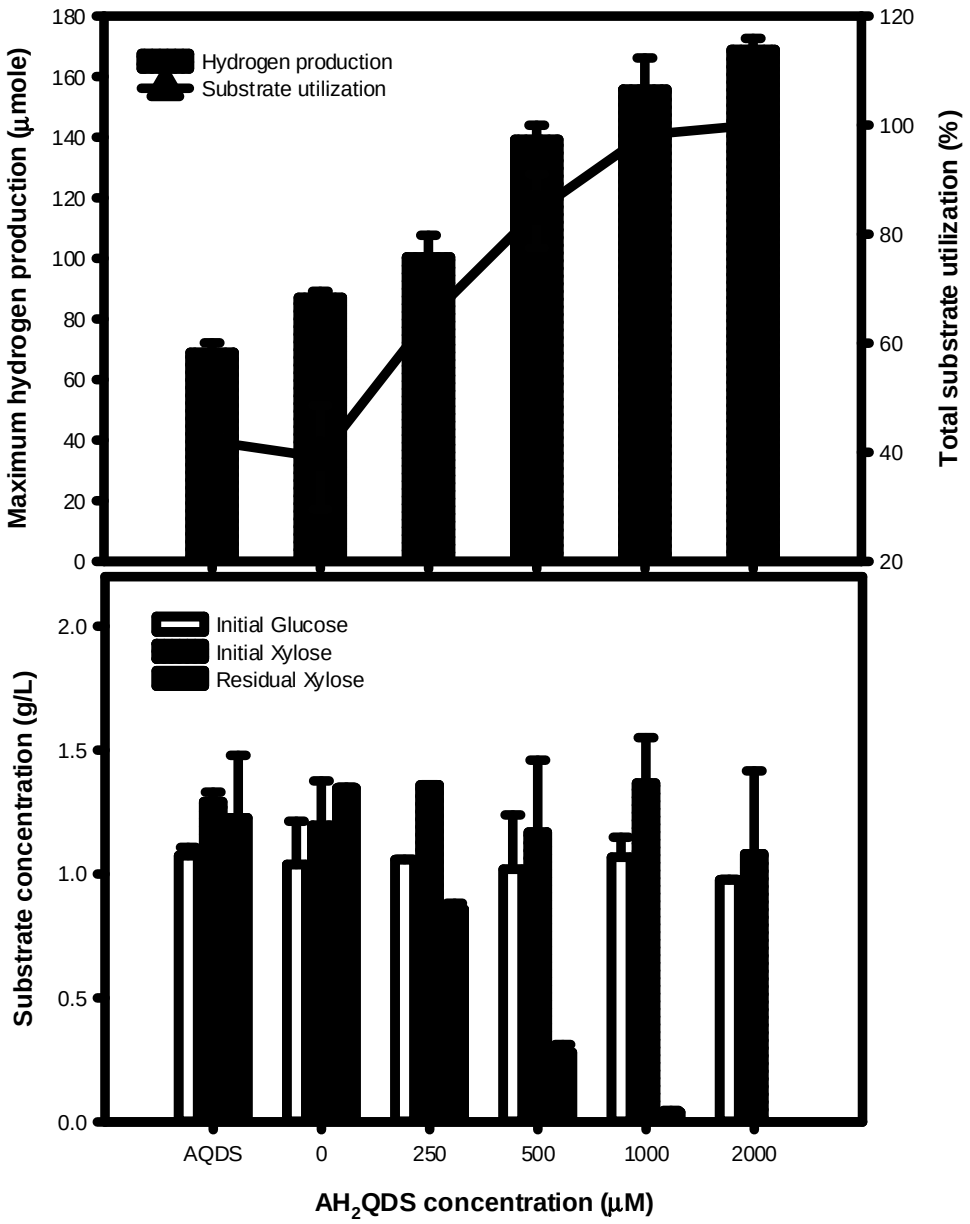

Figure 5.5. (a) Cumulative hydrogen production and total substrate utilization at different AH_2QDS concentrations. Total substrate utilization was calculated as total substrate consumed/initial total substrates. (b) Initial and residual substrate concentration in the batch experiments at different AH_2QDS concentrations. Residual glucose concentrations were not shown as they are below the detection limit of the HPLC-RI analysis.

CHAPTER 6 CONCLUSION AND FUTURE WORK

This research examines the influence of reduced electron shuttles (anthrahydroquinone disulfonate; AH_2QDS) on hydrogen production and substrate utilization in pure culture *Clostridium beijerinckii* fermentation. A novel physiological approach is proposed to increase the hydrogen production rate, molar yield, and substrate utilization by adding extracellular hydroquinones. Various carbohydrates associated with the lignocellulosic materials were tested as the substrates for fermentation.

When xylose was utilized as the primary substrate, extracellular hydroquinones increased the extent of xylose utilization by up to 56% and hydrogen yield by 24-37%. The increase of hydrogen yield was correlated to the pathway shift from butyrate production pathway to the acetate production pathway. Reduced electron shuttles shifted the carbon and electron flow from the butyrate pathway towards the acetate pathway, thereby generating "surplus" reducing equivalents for hydrogen production. An electron flow model was introduced to quantify the electrons transfer within the intracellular carrier pools, which provided evidence that more electrons from reduced ferredoxin were utilized for hydrogen production and fewer electrons were utilized to reduce NAD^+.

Adding reduced electron shuttles increased the hydrogen production kinetics when different substrates were tested, including glucose, xylose, and cellobiose. Adding AH_2QDS from 100 μM to 500 μM increased hydrogen production rates by up to 99%, 100% and 56% relative to unamended controls, when glucose, xylose, and cellobiose were utilized as the primary substrates, respectively. Adding reduced electron shuttles (AH_2QDS) also resulted in the increase of substrate utilization rates and biomass growth rates by up to 1.24 and 0.9 times, respectively. The coincidence of the increase of

hydrogen production rates, substrate utilization rates and apparent growth rates suggested that the increase of hydrogen production rates were possibly due to the global influence on the cellular metabolism. Results from resting cell suspension experiments with glyceraldehyde-3-phoshpate as the sole carbon source demonstrated that adding reduced electron shuttles increased the hydrogen evolution rate by 36-47% from glyceraldehyde-3-phoshpate. Since both the glycolysis (hexose metabolism) and pentose phosphate pathways (pentose metabolism) share the glyceraldehyde-3-phosphate as the intermediate, the increase of hydrogen evolution rates from glyceraldehyde-3-phoshapte might possibly contribute to the increases of the overall hydrogen production rates in the growth experiment when different substrates were utilized as the substrates for *Clostridium* fermentation.

Mixed sugars of glucose and xylose, the most two dominant carbohydrates in the hydrolysates of lignocellulosic materials, were investigated as the substrates for hydrogen production. Since different pretreatment and hydrolysis methods of diverse lignocellulosic feedstocks yield a wide range of glucose:xylose ratios, hydrogen production and substrate utilization were studied at different glucose:xylose ratios. Glucose was demonstrated to be a better substrate than xylose for *C. beijerinckii*, in terms of more efficient kinetics and more complete substrate utilization. Adding 250 μM reduced electron shuttles increased the extent of total substrate utilization and hydrogen production at glucose:xylose ratios of 1:1, 1:3 and 1:9. Glucose was completely fermented, and the increase of the total substrate utilization was due to the increase of xylose utilization. Adding 250 μM AH_2QDS also increased the specific hydrogen production rates and substrate utilization rates by 44-94% and 40-88%, respectively at

glucose:xylose ratios from 1:9 to 9:1. Increased hydrogen production and total substrate utilization, more specifically, xylose utilization were observed with increasing AH_2QDS concentrations from 250 μM to 2 mM at fixed glucose:xylose ratio of 1:1, which is common in the hydrolysates of different lignocellulosic biomass.

This research demonstrated that adding reduced electron shuttles increased the substrate utilization and hydrogen production in *C. beijerinckii* fermentation and opened the door for further optimization of the fermentation system with extracellular electron shuttles. One example is that we observed in a control experiment of this research that the oxidized electron shuttles addition increased butanol production. This direction is under investigation by several students in Finneran research group at University of Illinois and Clemson University. Another direction is to study the interaction between the fermenters and other respiratory microorganisms which can reduce electron shuttles using organic compounds. More work needs to be done to apply the findings from this research at the industrial level; however, it is possible to scale up the fermentation system and operate it in fed-batch or continuous system. Extracellular electron shuttles only cycle between oxidized form and reduced form without being consumed, which makes it possible to recycle the electron shuttles in the process. Organic acids that are considered byproducts of the fermentation processes can be used as the electron donors to reduce oxidized electron shuttles. Ideally, a two-stage process can be designed with the fermenters to generate hydrogen at the first stage and the respiratory microorganisms (i.e. *Geobacter metallireducens* and *Geobacter sulfurreducens*) at the second stage, which can use the organic acids from the fermentation process to reduce electron shuttles. It is also

interesting to investigate a system with electrodes, which could continuously provide the reducing power to reduce the extracellular electron shuttles.